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The Influence of DX-52-1 and Phosphorylation on the Interactions of Galectin-3 with its Binding Partners

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Matthew L. Rotondi, PhD

University of Connecticut, 2015

Galectin-3, a member of a large family of animal based β-galactoside-binding lectins has been shown to play a role in a number of cellular and pathogenic processes. This protein has been shown throughout the literature to interact with a diverse multiplicity of proteins, involved in a diverse array of cellular functions, including cell migration and cell proliferation, both of which are of particular in regards to cancer research. Covalent modifications to Galectin-3 that can undermine its binding partner interactions would definitely prove useful in such an enterprise.

DX-52-1, a semisynthetic an analog of the natural product quinocarcin, has been shown by the Fenteany Group, to demonstrate remarkable anti-migration and anti-proliferation properties with regard to a number of cancer cell lines. Furthermore this molecule has been shown to bind strongly and specifically to Galectin-3 and Radixin. In the course of this research DX-52-1 has been shown to modestly undermine a number of binding partner interactions of Galectin-3. It has also been demonstrated that the phosphorylation of Galectin-3 also influences its binding to DX-52-1. It has been further demonstrated that DX-52-1 may have an even more potent effect on the formation of higher order complexes that contain Galectin-3. This observation may serve to further explain the strong anti-migration and anti-proliferation effects of DX-52-1.

In the course of this work, efforts to map the binding site of DX-52-1 on Galectin-3 were undertaken. In the course of those efforts a novel method to narrow down potential binding sites was developed. Furthermore structure activity relationship studies were performed to determine the importance of certain functional groups on the binding properties of DX-52-1 in regard to Galectin-3 and Radixin as wells as its anti-cellular properties. The results of these SAR studies show a link between the binding of DX-52-1 to Galectin-3 and its anti-migration and anti-proliferation properties.

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Table of Contents

List of Figures

List of Equations

Introduction

The process through which a cell translocates itself from on position to another, is known as cell migration, an essential component to a number of biological processes both normal and pathogenic. Such processes include but are not limited to embryonic development, immunity, wound healing, angiogenesis, inflammation, tumor invasion, and metathesis (1, 2). In the case of immunity leukocytes must migrate into tissues in order to contend with infecting bacteria (3). Wound healing involves cells that surround the site of a wound and then migrate inward for the purpose of closing that wound (4).

Cell migration is arguably just as important to the progression of a cancer as is the ability of malignant cells to propagate uncontrollably. Cancerous cells that constitute a tumor like normal cells require blood vessels in order to continue propagating. Without blood vessels the size to which a tumor can grow is strictly limited (5). The process by which cells migrate from already existing blood vessels to a tumor in order to form new blood vessels is known as angiogenesis (5). It is through this process tumors are permitted to continue to grow and flourish. Cell migration is also critically important to the process of metathesis. This process involves cells breaking away from the primary tumor and migrating to nearby blood and/or lymphatic vessels in order to gain access to the circulatory system (6, 7). It is at this point the cancerous cells can easily make their way to other parts of the body and form any number of secondary tumors (5-7). Clearly angiogenesis and metathesis are critically important to the progression of cancers. Due to the fact that cell migration is an integral part of the aforementioned processes, it stands to reason the ability to inhibit the process of cell migration would useful in the development of therapeutic strategies.

The dynamics of the filamentous protein complexes that serve as a major component of the cytoskeleton, serve as the primary driving force of cell migration in multi-cellular organisms (2, 8). Actin is a 42 kD globular protein that exists in two primary states, the monomer G-Actin (globular) and the polymer F- Actin (filamentous) (2). The frequent interchange between these two states are responsible for a number of cellular processes including cell migration, cell signaling, organelle movement, cell division and muscle contraction (9). The process by which F-Actin is formed from G-Actinin (Figure 0-1) begins with the binding of ATP to G-Actin. These activated G-Actin can form oligomers, which have the potential to form nuclei, which initiate a rapid polymerization of other ATP bound G-Actin into F-Actin (9, 10). There are two distinct ends to the Actin filament, the barbed end and the pointed end. The Actin filament grows as it adds additional subunits to its barbed end, pushing against the cell membrane, and thus acts as the driving force of cell migration (8-10). The Actin subunits at the pointed end of the filament hydrolyze their bound ATP and as a result break away to be recycled allowing the cell migration process to be maintained (Figure 0-2). This overall process known as "treadmilling" can be inhibited either by molecules that interact with the Actin itself or by interacting with proteins that interact with the Actinin filaments directly or indirectly (2). Either approach has the potential of inhibiting cell migration which is important to the progression of cancers. This dissertation is concerned with the latter approach.

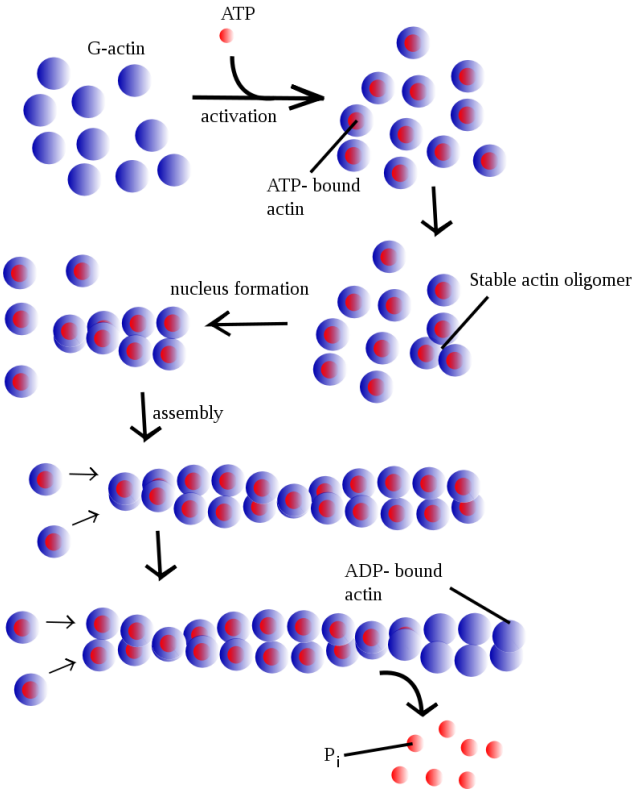

Figure 0-1: Actin Polymerization (http://en.wikipedia.org/wiki/Actin)

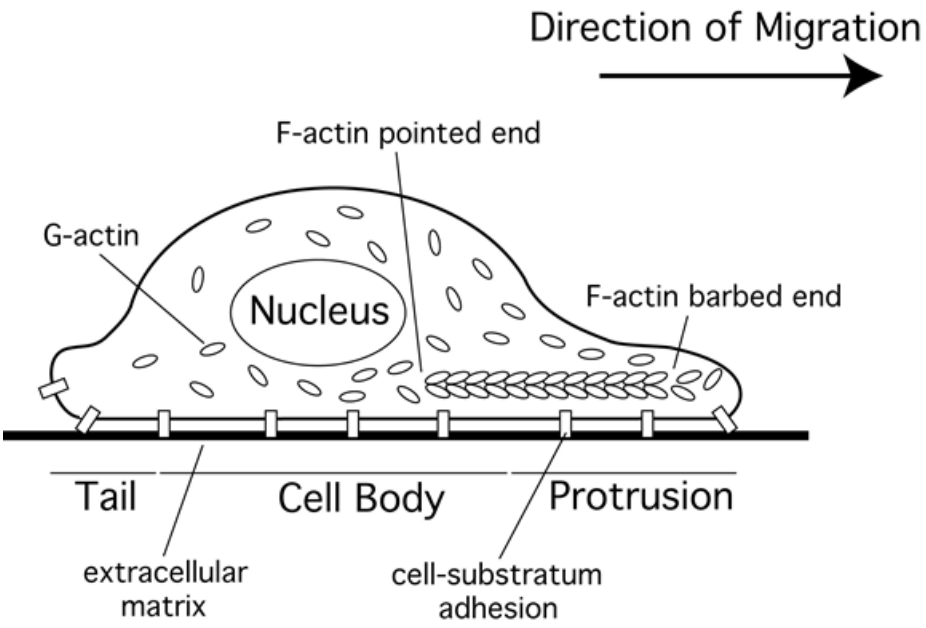

Figure 0-2: Animal cell migration propelled by the "treadmilling" of F-Actin (2)

There are a wide multitude of compounds that have been shown to inhibit cell migration (2). One such compound DX-52-1 (Figure 1-1) has been studied extensively by the Fenteany Group. DX-52-1 has demonstrated remarkable anti-proliferation and anti-migratory properties against a number of cancer lines (2, 11, 12). Though a series of pull down assays involving DX-52-1, the Fenteany Group was able isolate two proteins that strongly interact with DX-52-1. These proteins are Radixin and Galectin-3 (11, 12). This dissertation will focus on the relationship between Galectin-3 and DX-52-1. The reason for the selection of Galectin-3 as a focus of this work is that Galectin-3 has been demonstrated in the literature to interact with both β-Catenin (interacts with cell adhesion protein Cadherin) and α-Actinin (interacts with F-Actin), both of which play parts in cell migration (2, 12). Furthermore Galectin-3 has be reported extensively in the literature to interaction a wide multitude of proteins that affect a wide multitude of cellular processes (12-14). Elevated levels of Galectin-3 has also been linked to a number of different cancers (12-14).

This dissertation, the main body of which is divided into four chapters, will discuss research and observations concerning the interaction of DX-52-1 with Galectin-3 and the ability of DX-52-1 to influence the interaction between Galectin-3 and its protein binding partners. This dissertation will also describe effort to streamline the search for DX-52-1 binding site of Galectin-3. Furthermore this work will also discuss investigations involving the importance of certain functional groups of DX-52-1 in regard to its binding properties as well as its anti-cellular properties.

The first chapter will address two topics. The first topic involves the determination of the effect of the phosphorylation of Galectin-3 on the ability of the protein to bind DX-52-1. The second topic will involve the investigation the effects of Galectin-3 phosphorylation and the binding of DX-52-1 upon the ability of Galectin-3 to bind to each of ten protein binding partners.

These ten protein are involved in a diverse array of cellular processes. The effects of phosphorylation and the binding of DX-52-1 were both tested separately and together. The possible biological implications of the results described in the first chapter will also be discussed.

The second chapter will discuss two topics. One such topic will involve the observation of the effect of binding β-Catenin to Galectin-3 on its ability to subsequently bind DX-52-1. The effect α-Actinin binding to Galectin-3 upon its ability to bind DX-52-1 will also be examined. It stands to reason that the ability of DX-52-1 to affect cellular processes by binding to Galectin-3 maybe affected by the proteins Galectin-3 might already be interacting with. The other topic of the chapter concerns the formation of a three protein complex consisting of β-Catenin, Galectin-3, and α-Actinin. This complex is hypothesized to have Galectin-3 connecting β-Catenin to α-Actinin. This chapter will discuss experimental results supporting this hypothesis. The effect of DX-52-1 on the formation of this three protein complex will also be described and compared its effects on the binding properties of Galectin-3 discussed in the first chapter. The possible biological implications will also be addressed.

The third chapter will discuss the problem of direct determination of the DX-52-1 binding site via mass spectrometry. Experimental results that illuminate the cause of this problem will be discussed. Normally the alternative method would be the employment of alanine scanning via site directed mutagenesis. However a novel experimental method has streamlined what would normally be a much lengthier process. The novel method localizes the DX-52-1 binding site to a single tryptic fragment of Galectin-3 thus reducing the number of candidate amino acids that need to be subjected to alanine scanning. The development and experimental results of the novel method will be discussed.

The fourth chapter will discuss three topics. The first topic will involve the investigation of the effect of the modification of certain functional groups of DX-52-1 on its ability to bind to Galectin-3 as well as Radixin. The second topic of the chapter will involve measuring the effect of functional group modification on the ability of DX-52-1 to inhibit the migration of mammalian cells. The third topic will involve the observation of the effect of the modification of the functional groups of DX-52-1 upon its to inhibit cell proliferation.

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Chapter 1

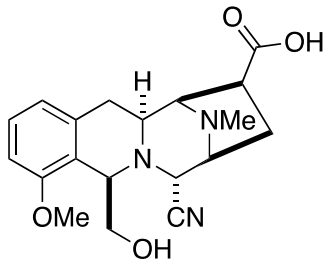
Interactions of Galectin-3 with its Binding Partners

Introduction

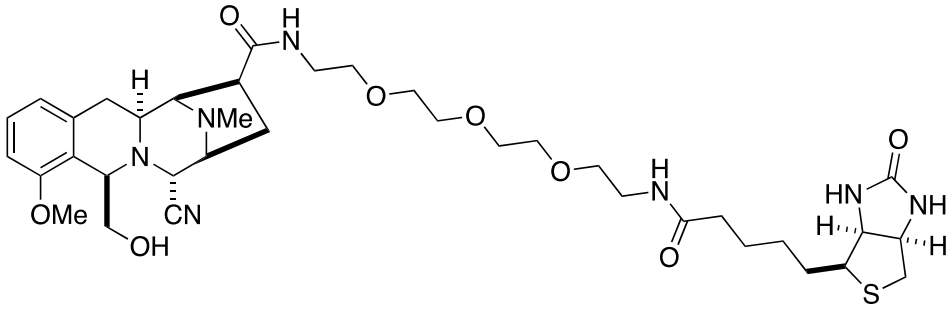
Galectin-3 is a 31-kDa protein that is a member of a large family of β-galactoside-binding lectins in animal cells (1-3). Galectin-3 has been classified as having several distinct structural domains: an N-terminal domain consisting of a 12 amino-acid segment that contains two casein kinase I phosphorylation sites, a repeated collagen-like sequence rich in proline, glycine, tyrosine and glutamine residues and a globular C-terminal half with a carbohydrate recognition domain (CRD) (1, 4). Galectin-3 plays a role in a broad range of cellular processes (2, 5) including pre-mRNA splicing, cell differentiation, cell adhesion to extracellular matrices, cell-cell adhesion, cell proliferation and cell motility (6, 7). Furthermore, Galectin-3 is involved in the regulation of apoptosis, oncogenesis and cancer cell metastasis (3, 10, 12). Galectin-3 interacts with a broad range of proteins of varied function (6, 7). Galectin-3 is found in the cytoplasm, nucleus, mitochondria, and associated to the cell membrane and extracellular spaces (6, 7). Functional dissection of Galectin-3's many interactions that are independent of carbohydrate binding interactions with other proteins or RNA is a pressing need in the field.

We have previously discovered that DX-52-1 (Figure 1-1), a semisynthetic analog of the tetrahydroisoquinoline natural product quinocarcin (also known as quinocarmycin), is an inhibitor of animal cell migration (7). DX-52-1 binds and inhibits functions of Galectin-3, as well as the membrane-cytoskeleton linker protein Radixin, through alkylation of specific amino acid residues on the proteins (7, 8). We then discovered that another tetrahydroisoquinoline, HUK-921, also

inhibits cell migration and has greater selectivity than DX-52-1 for Galectin-3 over radixin (8). DX-52-1 and HUK-921 bind to the CRD of Galectin-3 but are not competitive with the binding of β-galactosides, implying that these tetrahydroisoquinolines interfere with the interaction of Galectin-3 with proteins or other non-carbohydrate molecules (8). DX-52-1 and HUK-921 most likely act to inhibit the Galectin-3's ability to bind to its binding partners, either by direct steric hindrance or by an allosteric mechanism. DX-52-1 and related tetrahydroisoquinolines therefore represent potentially very important tools for understanding the functions of Galectin-3, particularly interactions with other proteins that may play a role in cell migration.



DX-52-1



BDX

Figure 1-1: Structures of DX-52-1 and Biotinylated DX-52-1 (BDX). BDX was prepared by EDC/NHS mediated coupling between DX-52-1 and biotin-PEG_3-amine, as previously described (Kahsai et al., 2006).

Galectin-3 has been shown to be phosphorylated on serine 6 and serine 12, and reversible phosphorylation appears to be an "on/off" switch regulating interactions between Galectin-3 and

a number of its binding partners (9-11). Casein kinase I phosphorylates Ser 6 (major) and Ser 12 (minor) (9). Because of the interaction of Galectin-3 with a multiplicity of proteins affecting a large number of cellular functions, the phosphorylation of Galectin-3 at Ser 6 and Ser 12 could have substantial effects on overall cell function and viability. For this reason, we chose to investigate the effect of Ser 6/Ser 12 phosphorylation of Galectin-3 upon several of its binding partners. These binding partners include Akt, Axin, Bcl-2, β-Catenin, CD40, Gemin-4, K-Ras, OCA-B, Sufu and TCF4 (1, 12-19). Akt is a serine/threonine kinase that is activated by Galectin-3 and participates with Galectin-3 in the control of apoptosis (19, 20). Axin is a negative regulator in the Wnt signaling pathway and plays a role in proteasome degradation (13, 21). Bcl-2 plays a role in the regulation of cellular apoptosis and has been found to have significant sequence similarity with Galectin-3 (22, 23). β-Catenin is part of the cadherin-containing adherens junction complex and plays a role in the Wnt signaling pathway (16, 24). CD40 plays a role in antigen presentation (17, 25). Gemin-4 plays a role in the splicing of pre-mRNA (14, 26). K-Ras has an effect upon both cell motility and expression of MMP-2 (12, 27-29) Sufu is a signaling protein that serves as a negative regulator of the Hedgehog signal transduction pathway (30, 31). OCA-B is a transcriptional co-activator involved in immune responses (15, 32). TCF4 functions as an immunoglobulin transcription factor (15, 34). We also looked at the combined effects of Ser 6/Ser 12 phosphorylation and DX-52-1 binding upon the interaction of Galectin-3 with its binding partners. Furthermore, we investigated the effect of the phosphorylation at Ser 6 and Ser 12 on the binding of DX-52-1 to Galectin-3.

Materials and Methods

Expression and Purification of Galectin-3

Human Galectin-3 was expressed as a GST fusion protein in the pGEX-2T-1 vector transformed into BL21 (DE3) *E. coli* cells. Small amounts of glycerol stock were removed by sterile 200 µl pipette tips to seed cultures in 40 ml of Luria-Bertani medium containing 1 mM ampicillin (LB-Amp). After growing the cells overnight at 37 °C, the culture was transferred to a flask containing 4LLB-Amp, followed by incubation at 37 °C until the absorbance at 600 nm was between 0.6 and 0.9. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated further at 37 °C for 5 h. The cells were then harvested by centrifugation (4,500 g for 10 min). The cell pellets were re-suspended in 140 ml of a lysis buffer consisting of 50 mM Tris (pH 7.5), 1 mg/ml lysozyme, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine and 1% Triton X-100 at 4 °C. The resuspended cells were homogenized and then incubated on ice for 30 min. The homogenate was centrifuged at 14,500 g for thirty min. A glutathione-agarose bead slurry (2 ml) was added to the lysate. The samples were rotated overnight at 4 °C. The beads were centrifuged at 4,500 g for 5 min and the lysate was discarded. The beads were re-suspended in 40 ml 50 mM Tris (pH 7.5) at 4,500 g for 5 min three times, with removal of supernatant and resuspension of beads between each spin. The beads were then resuspended in 8 ml of 50 mM Tris (pH 7.5) containing 10 units/ml thrombin. The sample was rotated overnight at 4°C. The samples were centrifuged at 4,500 *g* for 5 min, and the supernatant was transferred to a new tube. Protein concentration was determined by absorbance measurement at 280 nm (extinction coefficient of 33,710 M^{-1} cm^{-1} was estimated using "Richard's Protein Calculator" website). The supernatant was concentrated with a YM-10 Centricon filter until the final protein concentration reached 50

μM. The concentrated protein was then aliquoted and flash frozen by immersion in liquid nitrogen prior to storage at -80 ^{o}C.

Galectin-3 Binding Partner Plasmids

Plasmids expressing Galectin-3 binding partners as glutathione *S*-transferase (GST) fusion proteins were transformed into BL21 (DE3) *Escherichia coli* cells, with expression of the Galectin-3 binding partners as described above for Galectin-3. The cells were stored as glycerol stocks at -80 ^{o}C. Axin, Bcl-2, CD40, Gemin-4, OCA-B, Sufu and TCF4 were expressed from pGEX-2T-1 plasmids provided by W. Has (Columbia University), J. Teodoro (McGill University), F. Weiland (Heidelberg University), E. Briggs (Howard Hughes Medical Institute), R. G. Roeder (Rockefeller University), H. Miki (Osaka University) and Z. Yi (Ludwig Institute for Cancer Research), respectively. β-Catenin was expressed from a pGEX-4T-1 plasmid provided by Z.J. Sun (Stanford University). Akt was expressed from a pGEX-4T-2 plasmid provided by M. Rane (University of Louisville). K-Ras was expressed from a pGEX-5X-1 plasmid provided by M. Sammer (Bioinformatics Institute, A*STAR, Singapore).

Casein Kinase I-Catalyzed Phosphorylation of Galectin-3

Casein kinase I (CK1) was purchased from New England Biolabs. Reaction mixtures consisted of 20 μM Galectin-3, 300 μM ATP, 1X CK1 reaction buffer and 1,000 units of CK1. Control mixtures were identical to the reaction mixtures with the exception of the CK1 being omitted. Reaction and control mixtures were both incubated at 30 ^{o}C for 20 h.

Biotinylated DX-52-1 Binding Assays

Biotinylated DX-52-1 (BDX) was synthesized as previously described (8). On the first day CK1 and control reaction mixtures were prepared and incubated. On the second day BDX was added to both mixtures to a concentration of 900 μM. The mixtures were then incubated at 4 ºC for 27 h. On the third day equal volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer were added to each mixture. After boiling for 15 min, the mixtures were separated on a 12% polyacrylamide gel at 70 mA for 35 min before being transferred to polyvinylidene difluoride (PVDF) membrane at 200 mA for 1 h. The membrane was blocked with a solution of 5% milk in Tris-buffered saline-0.05% Tween 20 (TBS-T) at pH 7.6 for 1 h. Horseradish peroxidase (HRP)-linked anti-biotin antibody (Cell Signaling) was diluted 1,000-fold in milk/TBS-T and applied to the membrane for 1 h. The membrane was given three 15 min washes in TBS-T. Pierce Enhanced Chemiluminescence (ECL) Western Blotting Reagent was applied to the membrane for 5 min. The bands were visualized and their intensities quantified with Bio-Rad Quantity One software.

Galetin-3/Binding Partner Pull down Assays

Each Galectin-3 binding partner was expressed as a GST fusion protein in BL21 (DE3) *E. coli* cells. On the first day CK1 and control reaction mixtures were prepared and incubated for 20 h. Small amounts of glycerol stock were removed by sterile 200 μl pipette tips to seed 10 mL LB-Amp cultures. The cells are grown overnight at 37 °C. On the second day, each control mixture was divided into half. To the first half (designated “Cont”), DMSO was added to a concentration of 1% by volume. To the second half (designated “DX”), DX-52-1 was added (as a solution in DMSO) to a concentration of 900 μM. The CK1 reaction mixture was also divided in half. To the

first half of the reaction mixture (designated “Phos”), DMSO was added to a concentration of 1%. To the second half of the reaction mixture (designated “DX-52-1/Phos”), DX-52-1 was added to a concentration of 900 μM. Cont, DX, Phos and DX-52-1/Phos were each incubated at 4 ^{o}C for 20 h.

On the third day, the beads were centrifuged at 4,500 *g* for 5 min, and the lysate was discarded. The beads for each binding partner were resuspended in 15 ml 50 mM Tris (pH 7.5), centrifuged at 4,500 g for 5 min and the supernatant was discarded. The aforementioned step was repeated twice. Each set of beads was resuspended in 1 ml 50 mM Tris (pH 7.5), (in the case of K-Ras, 10 mM GTP was included). For each assay an equal volume of glutathione beads bearing a GST-binding partner fusion protein was added to the Cont, DX, Phos and DX/Phos samples. The mixtures are rotated for 20 h at 4 ^{o}C.

On the fourth day, the mixtures were centrifuged, and the supernatants discarded. The beads were washed three times with 50 mM Tris (pH 7.5), with final volumes kept minimal. 40 μl SDS-PAGE loading buffer was added to each set of beads. After boiling for 15 min, the mixtures were then separated on a 12% polyacrylamide gel before being transferred to a PVDF membrane. The membrane was blocked with a 5% milk/TBS-T solution for 1 h. Mouse antiGalectin-3 (Santa Cruz Biotechnology) was diluted 1,000 fold in 5% milk/TBS-T and applied to the membrane for 1 h. The membrane was washed three times with TBS-T for 15 min per wash. Goat anti-mouse-HRP was diluted 10,000-fold in 5% milk/TBS-T and applied to the membrane for 1 h. The membrane was given three 15 min washes in TBS-T. Pierce ECL Western Blotting Reagent was then applied to the membrane for 5 min. The bands were visualized and their intensities quantified with Bio-Rad Quantity One software.

Results and Discussion

The structures of DX-52-1 and BDX are shown in Figure 1-1. The BDX binding assay shows that Ser 6/Ser 12 phosphorylation of Galectin-3 has a very substantial effect on the binding of DX-52-1 upon the protein (Figure 1-2). Galectin-3 phosphorylated in such a manner shows increased binding of DX-52-1 by 320% ± 102% (mean ± SEM), as derived from the data displayed in Figure 1-2. There appear to be two possible explanations for this dramatic increase in DX-52-1 binding. The first possibility is that Ser 6/Ser 12 phosphorylation of Galectin-3 by CK1 increases the on rate or decreases the off rate for DX-52-1 binding at a single site. The ratio of BDX binding between phosphorylated and unphosphorylated Galectin-3 is more or less constant at 290% ± 14% (Figure 1-3), which suggests that there is no change in the kinetics of modification upon phosphorylation. The second possibility is that Ser 6/Ser 12 phosphorylation changes the conformation of Galectin-3 in such a manner that additional DX-52-1 binding site(s) become accessible. This would result in each Galectin-3 molecule binding multiple BDX molecules, thus leading to the observed increase in BDX signal. Due to the fact that the ratio of BDX binding remains constant the first possibility is rendered unlikely leaving us with the second possibility.

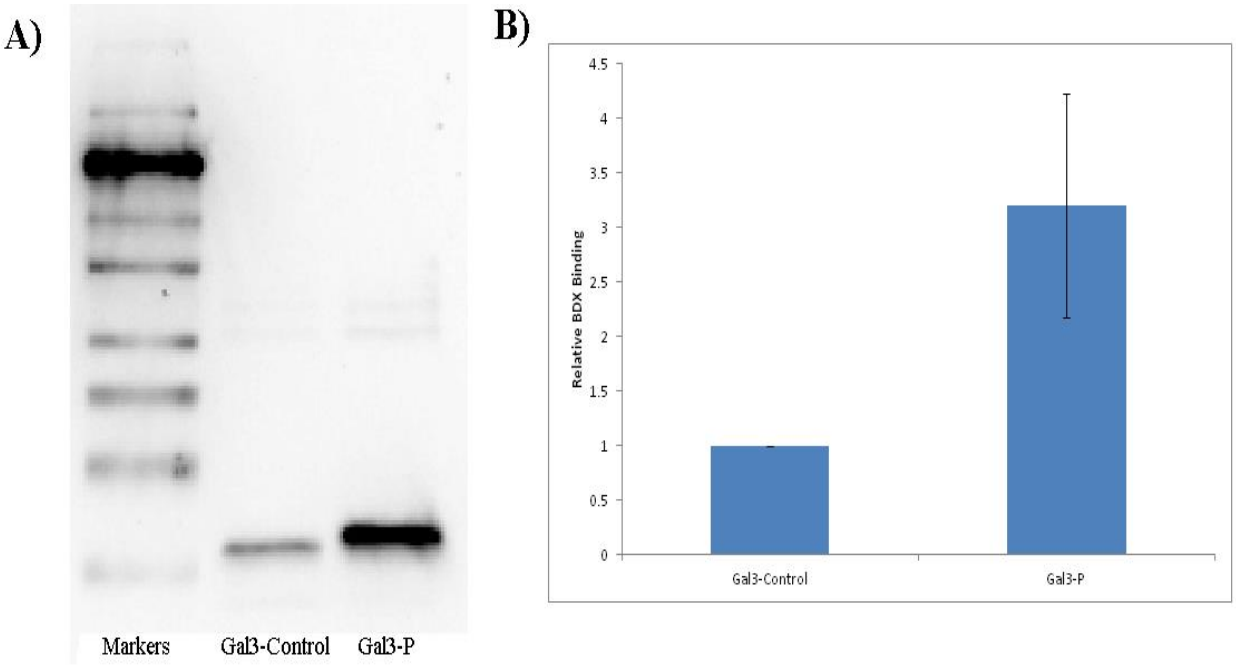


Figure 1-2: Effect of Phosphorylation on Galectin-3 on BDX Binding

A) Western Blot of Biotinylated DX-52-1 Binding Assay. Lane 1, biotinylated molecular weight markers; lane 2, galectin-3 control; lane 3, phosphorylated galectin-3

B) Comparison of the Relative Intensity of the BDX signals for Galectin-3 (Gal3-Control) and Phosphorylated Galectin-3 (Gal3-P). Relative Intensity is defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Gal3-Control Sample (SD, n = 3). Difference between Gal3-Control and Gal3-P was statistically significant (p = 0.0054).

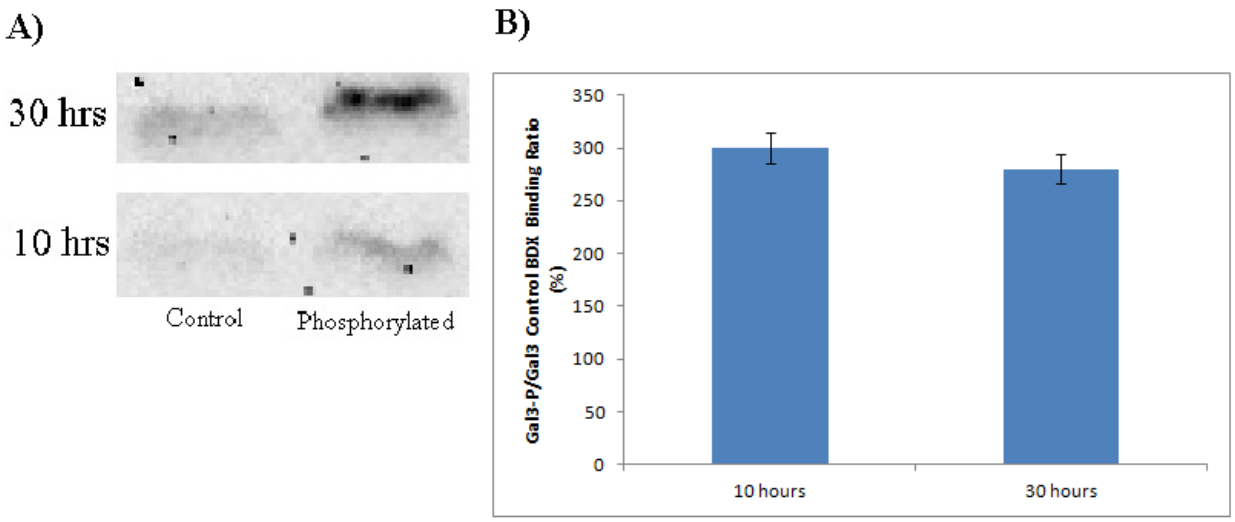


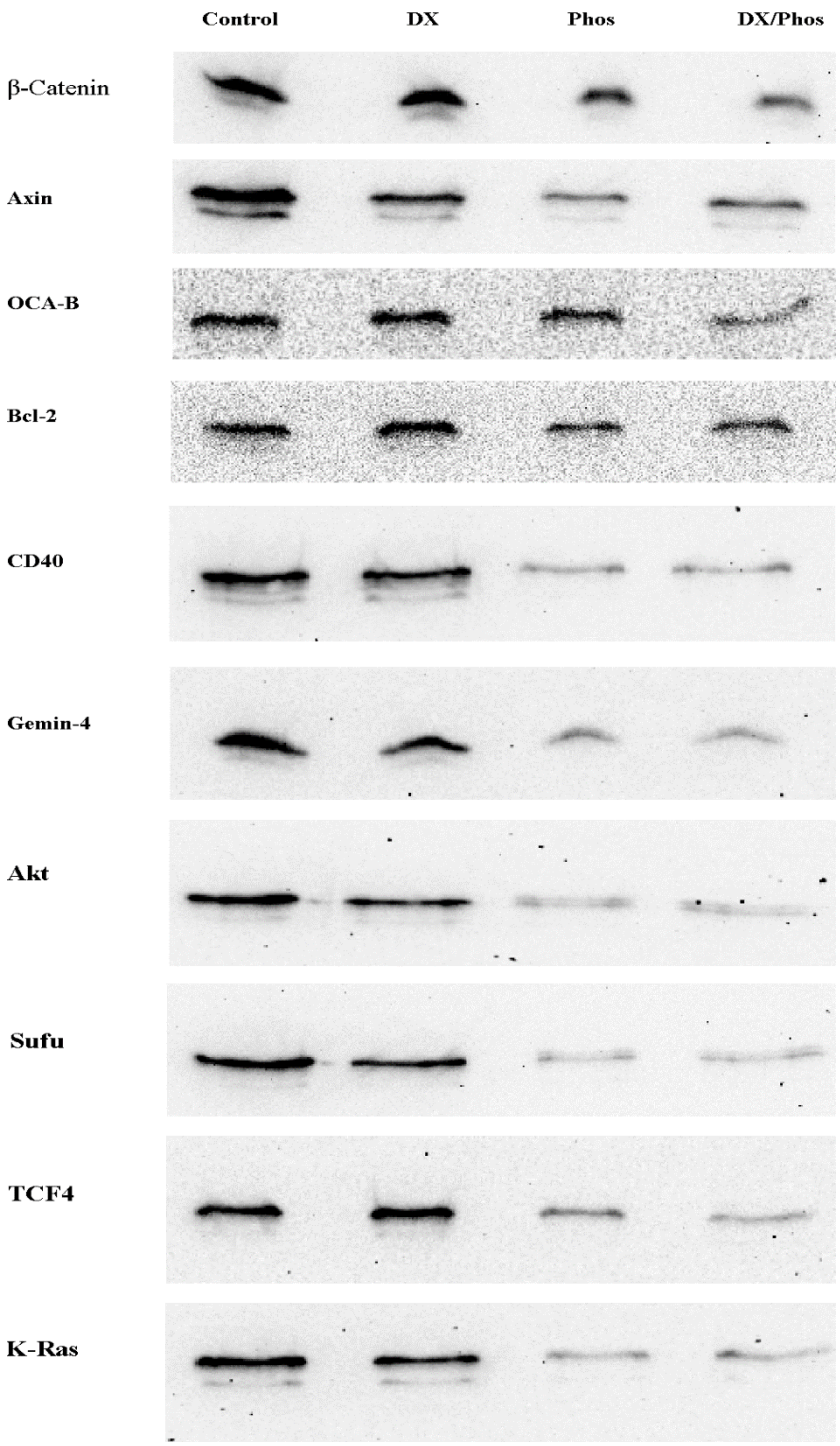
Figure 1-3: Comparison of BDX Binding Reaction Galectin-3 Control versus Phosphorylated Galectin-3 at 10 hours and 30 hours

A) Western Blot of Biotinylated DX-52-1 Binding Assay for Galectin-3 Control and phoshorylated Galectin-3 at 10 hours and 30 hours
B) Comparison of Ratios of BDX Signal Intensity between Phosphoryalted Galectin-3 and Galectin-3 at 10 hours and 30 hours. Difference between 10 and 30 hours was not statistically significant

Binding of DX-52-1 to Galectin-3 resulted in a statistically significant reduction in the interactions of Galectin-3 with six of the Galectin-3 binding partners tested (Figure 1-4). The strongest reduction by DX-52-1 was found for the interaction of Galectin-3 with Axin, which was reduced to 54.5% ± 5.90% of the control. The interaction of Galectin-3 with Akt was reduced to 74.8% ± 13.5% of the control. The interaction of K-Ras and Galectin-3 was reduced to 76.1% ± 11.3% of the control. The interaction between Galectin-3 and Gemin-4 was reduced to 77.2% ± 12.9% of the control. The interaction between Galectin-3 and Sufu was reduced to 77.2% ± 10.4% of the control. DX-52-1 reduced the interaction of Galectin-3 with OCA-B to 84.5% ± 5.90% of the control. Although these interactions are not completely disrupted by DX-52-1, collectively, these modest reductions in Galectin-3's ability to interact with these binding partners could cause

a substantial effect on the physiology of the cell. Since six out of ten of the binding partners tested were negatively affected in their ability to bind Galectin-3 by DX-52-1, it is likely that DX-52-1 also inhibits the binding of other Galectin-3-binding proteins to Galectin-3. Moreover, the results imply that these six proteins interact with Galectin-3 along an overlapping surface of the protein that encompasses the DX-52-1-binding site, assuming that the DX-52-1 sterically blocks binding of these Galectin-3-binding proteins. It also implies that, conversely, the remaining four binding partners tested bind to another part of Galectin-3 outside of the DX-52-1 binding site. Alternatively, however, DX-52-1 could cause a conformational change in Galectin-3 that allosterically affects binding of some but not all galecitn-3-binding proteins. In either case, the results raise interesting and testable hypotheses about how these different Galectin-3-binding proteins interact with Galectin-3. They also, of course, provide a framework for further studies on the molecular mode of action of DX-52-1.

A


Control
DX
Phos
DX/Phos
β-Catenin
Axin
OCA-B
Bcl-2
CD40
Gemin-4
Akt
Sufu
TCF4
K-Ras

B

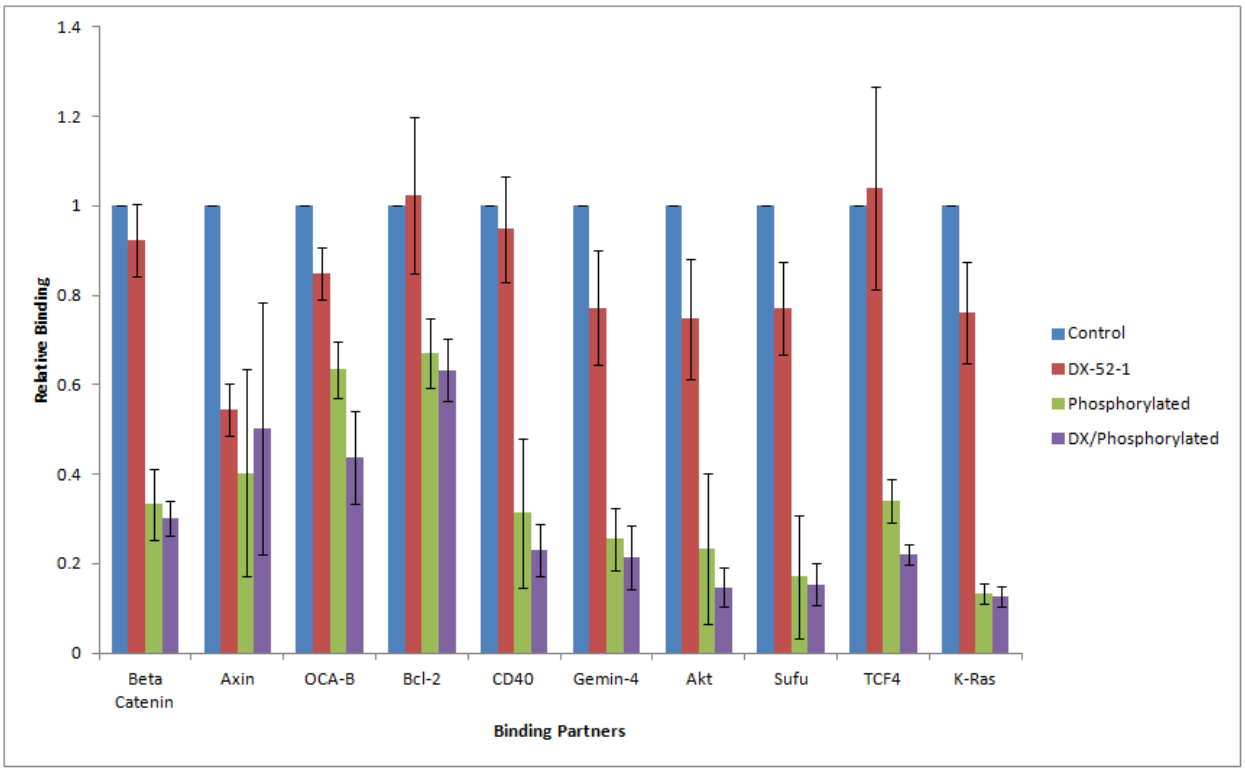


Figure 1-4: Effects of DX-52-1 and Phosphorylation on Galectin-3 Interaction with Binding Partners

A) Western Blots of Galectin-3/Binding Partner Co-Precipitation Assays. Each row shows the interactions between a binding partner and galectin-3 that has been unmodified (first column [control]), modified by DX-52-1 (second column), phosphorylated (third column) or both modified by DX-52-1 and phosphorylation (fourth column). Blots were probed with mouse anti-galectin-3 antibody and goat anti-mouse antibody conjugated to HRP.

B) Relative Intensity of Interaction of Galectin-3 to Binding Partners. Relative intensity was defined as the ratio between experimental sample intensity and control sample intensity (SD, $n = 3$). DX-52-1 had a statistically significant effect ($p < 0.005$) for binding of galectin-3 to Axin, OCA-B, Gemin-4, Akt, and Sufu. Phosphorylation of galectin-3 had a statistically significant effect for all ten binding partners ($p < 0.01$ in all cases). Finally, DX-52-1 treatment resulted in reduced binding of phosphorylated galectin-3 OCA-B and TCF4 ($p < 0.05$).

The interactions between Galectin-3 and all ten of the binding partners tested was affected by phosphorylation at Ser 6/Ser 12 (Figure 1-4). Phosphorylation at Ser 6/Ser 12 reduced the interaction between Galectin-3 and K-Ras to 13.3% ± 2.30% of the control. The interaction between Sufu and Galectin-3 was reduced by phosphorylation to 17.1% ± 13.7% of the control. The interaction between Galectin-3 and Akt was reduced by phosphorylation to 23.4% ± 16.7% of the control. The interaction of phosphorylated Galectin-3 with Gemin-4 is 25.5% ± 7.00% of the control. The interaction between Galectin-3 and CD40 was reduced by phosphorylation to 31.4% ± 16.7% of the control. Phosphorylation reduced the binding of Galectin-3 to β-Catenin to 33.3% ± 8.00% of the control. Phosphorylation of Galectin-3 reduced the interaction between the protein and TCF4 to 34.0% ± 5.00% of the control. Phosphorylation reduced the binding of Galectin-3 to Axin to 40.3% ± 23.3% of the control. The interaction of phosphorylated Galectin-3 with OCA-B was 43.7% ± 6.30% with regard to the control. Phosphorylation reduced the binding of Galectin-3 with Bcl-2 to 67.0% ± 7.80% of the control. Since the binding of all ten of the Galectin-3-binding proteins to Galectin-3 was reduced by phosphorylation of Galectin-3, it is likely that this is a very broad mechanism of negative control binding of Galectin-3 to other proteins. Furthermore it can be inferred that compared to DX-52-1 binding, phosphorylation at Ser 6/Ser 12 affects Galectin-3 interaction with its binding partners much more potently. This due to the observation that all binding partners tested showed reduced interaction with phosphorylated Galectin-3 and the reductions tended to be of a considerably higher magnitude those observed in the case of DX-52-1. It can be speculated that if a way to stimulate phosphorylation of Ser 6/Ser 12 in vivo can be devised than such a way might prove even more promising than DX-52-1 derived anticancer therapeutic strategies. We observed no statistically significant Ser 6/Ser 12 phosphorylation on the ability of DX-52-1 to inhibit interaction of Galectin-3 with its binding partners, except in the case

of OCA-B and TCF4 (Figure 1-4). The interaction between phosphorylated Galectin-3 and OCA-B is reduced to 70.2% ± 21.4% by DX-52-1 versus the reduction to 84.8% observed with non-phosphorylated Galectin-3. The interaction between phosphorylated Galectin-3 and TCF4 is reduced to 65.4% ± 5.50% by DX-52-1 versus the complete lack of statistically significant difference in the binding of non-phosphorylated Galectin-3. These results imply that only in the case of these two proteins are there additive or synergistic effects of the two inhibitory modifications of Galectin-3: the "natural" phosphorylation of Ser 6/Ser 12 on Galectin-3 by CK1 and the "unnatural" alkylation of Galectin-3 by DX-52-1. In the other cases where DX-52-1 inhibits the binding of Galectin-3-binding partners (Akt, Axin, Gemin-4, K-Ras and Sufu), the inhibitory effect of phosphorylation swamps out the inhibitory effect of DX-52-1 on binding of Galectin-3 to its binding partners. Since the precise binding site or sites of DX-52-1 have not yet been mapped, it is possible that DX-52-1 alkylates Ser 6 and/or Ser 12, and therefore is competitive with phosphorylation at these residues. On the other hand, DX-52-1 may alkylate entirely different residues. That investigation, however, is beyond the scope of the present study.

In summary, our results suggest that DX-52-1 acts by inhibiting specific interactions of Galectin-3 with its binding proteins along the discrete surfaces of Galectin-3. Phosphorylation has a general negative effect on interactions of Galectin-3 with its binding partners. It is clear that interactions of Galectin-3 with other proteins can be modulated endogenously by phosphorylation and artificially by small molecules. DX-52-1 and presumably its congeners remain the only known small-molecule inhibitors of the interaction of Galectin-3 with Galectin-3-binding proteins. They therefore have considerable potential as tools for the study of Galectin-3 function and as therapeutic agents for diseases involving Galectin-3 such as oncogenesis.

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Chapter 2

Effect of DX-52-1 on Formation of β-Catenin/Galectin-3/α-Actinin Complex

Introduction

As previously stated Galectin-3 has been demonstrated to perform a critical role in numerous cell functions and pathogenic processes (1-5). These functions include but are by no means limited to cell motility, cell proliferation, cell to cell adhesions, cell adhesion to extracellular matrices, cell differentiation, and pre-mRNA splicing (1-3, 5-8). Galectin-3 has furthermore been shown to play pivotal roles in cellular apoptosis regulation, metathesis of cancerous cells, as well as oncogenesis (3, 9, 10). Furthermore Galectin-3 has been detected in a multitude of cellular locations which include the nucleus, cytoplasm, mitochondria, cell membrane, and even the extracellular spaces (3, 6-8, 11, 12). Galectin-3 has also been shown to bind with a diverse multitude of proteins including β-Catenin, OCA-B, K-Ras, and many others (13-17). In Chapter 1 it has been demonstrated that certain covalent modifications undermine the interactions between Galectin-3 and ten of the aforementioned binding partners. It stands to reason that a covalent modification that could undermine the interaction between Galectin-3 and its various binding partners would likely affect a diverse number of cellular processes. This chapter concerns an investigation of the interactions of Galectin-3 with β-Catenin and α-Actinin, both separately and simultaneously, due to the fact that these two binding partners respectively play roles in the in the interaction of the cell surface with the Actinin cytoskeleton, which can have an effect on cell moltility and metathesis (13, 18-20).

β-Catenin is a 92 kDa protein that is a member of the Catenin family of proteins. It is both a subunit of the cadherin protein complex and has been shown to play a role in the Wnt signaling pathway (13, 20-23). β-Catenin has been shown to play a role in the developmental systems of animals as a signal transduction molecule (21). The deregulation of β-Catenin signaling has been shown to a significant role in oncogenesis (23). β-Catenin is known to interact with F-actin binding protein α-Actinin though α-Catenin, illustrated in Figure 2-1 (24). However in the course of our work we have discovered through a series of pull down assays that α-Actinin can bind galectin-3. This led us to consider the possibility that β-Catenin, an established binding partner of Galectin-3 might bind to α-Actinin through its binding to Galectin-3 as is the case with α-Catenin (24, 25).

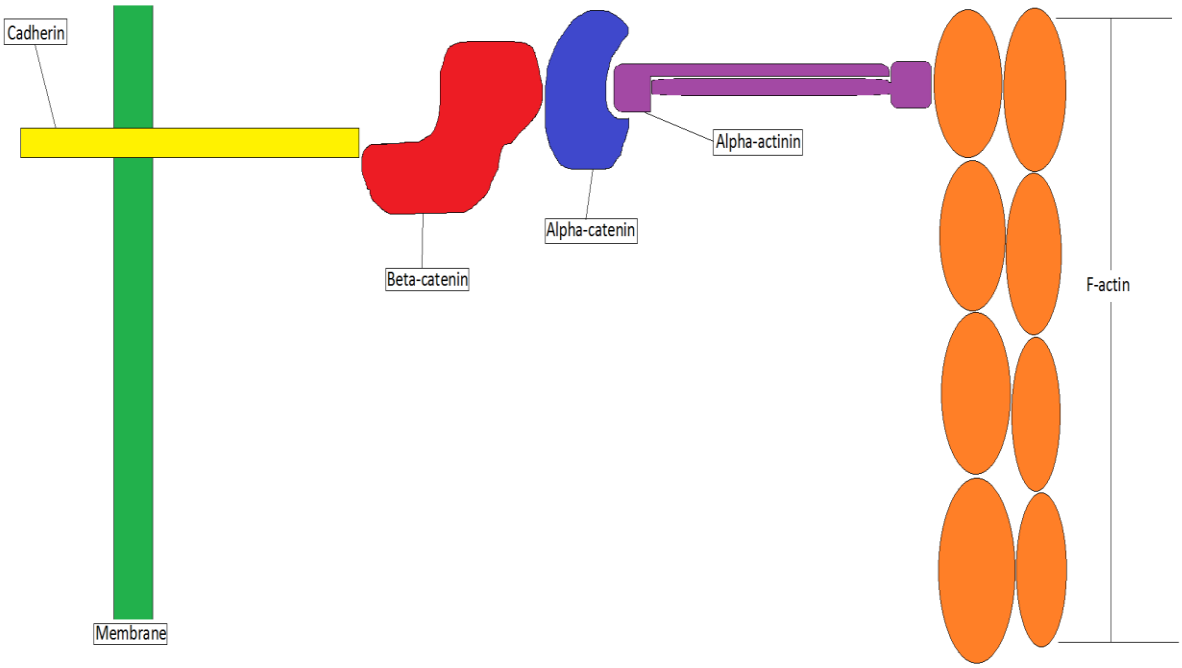

Figure 2-1: Interaction between β-Catenin and α-Actinin though α-Catenin providing a link between cell adhesion protein Cadherin and cytoskeletal protein complex F-actin

α-Actinin is 108 kDa protein that binds to the Actinin cytoskeleton and forms an anti-parallel dimer with itself (18). This protein serves the cytoplasmic face of a number of cell adhesion sites either by binding to the cytoplasmic domain of transmembrane protein such as the

β subunit of Intergrin or indirectly as is the case for the interaction of α-Actinin with Catherin though its binding to α-Catenin, which in turn binds to β-Catenin, which binds to the C-terminal side of Catherin as illustrated in Figure 2-1 (18, 26-28). Due the binding of the Actinin cytoskeleton by α-Actinin it plays an essential role in cell motility. In the course of this work we have studied the interaction between α-Actinin and a protein complex comprising β-Catenin and Galectin-3. We have furthermore studied the ability of DX-52-1 to interfere with the aforementioned interaction.

DX-52-1 (Figure 1-1) is an analog of quinocarcin that has been shown as an inhibitor of cellular migration, a key part of the metastatis of tumor cells (29-31). It has been determined that DX-52-1 targets both Radixin and Galectin-3 (30, 31). DX-52-1 has also been demonstrated to have a strong effect on cellular proliferation and migration (30, 31). The suggested mechanism by which DX-52-1 attaches to its targets it the disassociation of its nitrile group followed by the nucleophilic attack of the resulting carbocation by a nucleophilic amino acid side chain of the targeted protein (30). Galectin-3 has been revealed to be one of the specific targets of DX-52-1 by method of a pull down assay involving BDX, (Figure 1-1) and strepadvidin beads (31). Due to the fact that Galectin-3 interacts with a multitude of proteins that mediate the interaction between cells and the extracellaur matrix as well as other cells, the inhibition of the cell migration via compound that binds galectin-3 would be expected (7, 8). The most basic explanation for such an effect being brought about by DX-52-1 and other small molecules is the direction reduction of the interaction between Galectin-3 and its binding partners. This might occur though the steric hindrance of binding partner attachment or via a conformational shift (allostery) in Galectin-3 that reduces its interaction with its binding partners. In the course of our work we have discovered through a series of pull down assays of Galectin-3 by an extensive series of its binding partners that DX-52-1 does

reduce the binding of Galectin-3 in a number of cases but the reductions although statistically significant, are modest (unpublished results). In an effort to explain the strong effect of DX-52-1 on cellular proliferation and migration detected by Kahasi et al we had hypothesized that due to the fact that this list Galectin-3 binding partners are associated with a diverse number of cell functions, reductions of several interactions simultaneously could have a strong cumulative effect on overall cell function and viability. However the recent findings that will be described in this work have lead us to formulate a second hypothesis.

In this work we attempt to determine effect of DX-52-1 upon the formation of a three protein complex consisting of β-Catenin, Galectin-3, and α-Actinin. Furthermore we will also endeavor to use data concerning the effects of β-Catenin and α-Actinin upon the binding of BDX to make a rough determination of where on Galectin-3 DX-52-1 bind in relation to where β-Catenin and α-Actinin bind. We will also attempt to deduce the nature of the effect of DX-52-1 on the interactions of Galectin-3 with β-Catenin and/or α-Actinin.

Materials and Methods

Expression and Purification of β-Catenin and Galectin-3

Human β-Catenin was expressed as a GST fusion protein by a pGEX-2T-1 vector (donated by Z.J. Sun of Stanford University) transformed into BL21 (DE3) *E. coli* cells. Small amounts of glycerol stock were removed by sterile 200 µl pipette tips to seed cultures in 40 ml of Luria-Bertani medium containing 1 mM ampicillin (LB-Amp). After growing the cells overnight at 37 °C, the culture was transferred to a flask containing 4 l LB-Amp, followed by incubation at 37 °C until the absorbance at 600 nm was between 0.6 and 0.9. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated further at 37 °C for 5

h. The cells were then harvested by centrifugation (4,500 g for 10 min). The cell pellets were stored overnight at -80 °C. On the next day the cell pellets were thawed at 4 °C over a period of three hours. The cell pellets were then re-suspended in 140 ml of a lysis buffer consisting of 50 mM Tris (pH 7.5), 1 mg/ml lysozyme, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine and 1% Triton X-100 at 4 °C. The resuspended cells were homogenized and then incubated on ice for 30 min. The homogenate was centrifuged at 14,500 g for thirty min. Glutathione-agarose bead slurry (2 ml) was added to the lysate. The beads were rotated overnight at 4 °C. The beads were centrifuged at 4,500 g for 5 min and the lysate was discarded. The beads were re-suspended in 40 ml 50 mM Tris (pH 7.5) at 4,500 g for 5 min three times, with removal of supernatant and resuspension of beads between each spin. The beads were then resuspended in 8 ml of 50 mM Tris (pH 7.5) containing 10 units/ml thrombin. The beads were rotated overnight at 4°C. The beads were centrifuged at 4,500 g for 5 min, and the supernatant was transferred to a new tube. Protein concentration was determined by absorbance measurement at 280 nm (extinction coefficient of 61,950 M^{-1} cm^{-1} was estimated using "Richard's Protein Calculator" website). The supernatant was concentrated with a YM-10 Centricon filter until the final protein concentration reached 40 μM. The concentrated protein was then aliquoted and flash frozen by immersion in liquid nitrogen prior to storage at -80 ^{o}C. Galectin-3 was prepared using the protocol described in Chapter 1.

In order to prepare β-Catenin Bearing Beads for use in the pull down assays that are described later in this section, the protocol is followed up to the step involving the three washes with 50 mM Tris (pH 7.5). Thereafter the beads are re-suspended in 50 mM Tris (pH 7.5) to a volume of 1 ml. The beads are then stored at 4 ^{o}C until their later use.

Expression and Purification of α-Actinin

Human α-Actinin was expressed as a histidine tagged protein by a pRSET-6-His-2 vector transformed into BL21 (DE3) *E. coli* cells. Small amounts of glycerol stock were picked to seed a culture in 10 ml of Luria-Bertani medium containing 1 mM ampicillin (LB-Amp). After growing the cells overnight at 37 °C, the culture was transferred to 1 L LB-Amp followed by incubation at 37 °C until the OD_{600} was between 0.6 ~ 0.9. IPTG was added to a final concentration of 1 mM and the culture was further incubated at 37 °C for 5 hours. The cells were then harvested by centrifugation (4,500 g for 10 minutes). The cell pellets were resuspended in cold 35 ml lysis buffer containing 50 mM NaH_2PO_4 (pH 7.5), 500 mM NaCl, 20mM imidazole, 1 mg/ml lysozyme, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, and 1% Triton X-100. The re-suspended cells were homogenized then incubated on ice for 30 minutes. The homogenate was then centrifuged at 20,000 g for 30 minutes and the lysate was retained. Ni-NTA agarose (500 μl) was added to the lysate which was then rotated overnight at 4°C. The beads were centrifuged at 5,000 rpm for 5 minutes and the lysate was discarded. The beads were resuspended in 10 ml His Tag Binding Buffer containing 50 mM NaH_2PO_4 (pH 7.5), 500 mM NaCl and 20 mM imidazole and centrifuged at 4,500 g for 5 minutes with the supernatant discarded. The aforementioned step was repeated twice. The beads were resuspended in 3 ml His Tag Elution Buffer containing 50 mM NaH_2PO_4 (pH 7.2), 500 mM NaCl and 500 mM imidazole. The beads were vortexed and rotated overnight at 4 °C. The next day the beads were centrifuged at 4,500 rpm for 5 minutes and the supernatant was retained. The concentration of the protein was determined by UV absorbance measurement at 280 nm (extinction coefficient of 124,320 M^{-1} cm^{-1} was estimated using "Richard's Protein Calculator" website). The supernatant was concentrated

by YM-10 centricon until the final protein concentration reached 25 μM. The concentrated protein was then aliquoted and flash frozen by immersion in liquid nitrogen prior to storage at -80 ^{o}C.

In order to prepare α-Actinin Bearing Beads for use in the pull down assays that are described later in this section, the protocol is followed up to the step involving the three washes with His Tag Binding Buffer. Thereafter the beads are re-suspended in His Tag Binding Buffer to a volume of 1 ml. The beads are then stored at 4 ^{o}C until their later use.

Pull Down Assay of Galectin-3/β-Catenin Complex by α-Actinin

On the first day a binding mixture of 1 μM β-Catenin and 5 μM Galectin-3 as well as a blank mixture of 1 μM β-Catenin alone was incubated overnight at 4 ^{o}C. On the second day the binding mixture and the blank mixture was diluted 10X in water. Equal volumes of α-Actinin bearing beads (Preparation described earlier in this section) were added to each mixture. These mixtures were then rotated overnight at 4 ^{o}C. On the third day the beads in the binding and blank mixtures were spun down and the supernatants discarded. The beads were washed three times with His Tag binding buffer with final volumes being minimized. 40 μl SDS-PAGE loading buffer is added to each set of beads. After boiling for 15 minutes the mixtures were then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Rabbit anti β-Catenin purchased from Sigma-Aldrich^{TM} was diluted 1,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. The membrane was washed three times with TBS-T, 15 minutes per wash. Goat antirabbit-HRP was diluted 10,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. Once again the membrane was washed three times with TBS-T, 15 minutes

per wash. Pierce™ ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One™.

Galectin-3 Binding Assays with β-Catenin and α-Actinin

Two reaction mixtures of Galectin-3 were prepared. The first mixture consisted of 2 μM Galectin-3 and 1% DMSO and served as the control mixture. The second mixture consisted of 2 μM Galectin-3 and 1 mM DX-52-1. The reaction mixtures were incubated overnight at 4 ºC. The mixtures were diluted 30-fold in water. Equal volumes of β-Catenin bearing glutathione beads (preparation described earlier in this section) were added to the reaction mixtures which were then rotated overnight at 4 ºC. The beads were then washed three times with 50 mM Tris (pH 7.5). The beads were then spun down and the supernatants discarded. The beads are washed three times 50 mM Tris (pH 7.5) with final volumes being minimized. 40 μl SDS-PAGE loading buffer is added to each set of beads. After boiling for 15 minutes the mixtures were then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Mouse antigalectin-3 purchased from Santa Cruz Biotechnology™ was diluted 1,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. The membrane was washed three time with TBS-T, 15 minutes per wash. Goat antimouse-HRP was diluted 10,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. Once again the membrane was washed three time with TBS-T, 15 minutes per wash. Pierce™ ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One™.

The Galectin-3 Binding Assay with α-Actinin bearing beads (Preparation described earlier in this section) was performed in a nearly identical matter as it was to β-Catenin with only two modifications. The first modification was the beads were allowed to settle by gravity instead using a centrifuge. The second modification was that the washes were performed with His Tag Binding Buffer in place of Tris.

BDX Binding Assays

Biotinylated DX-52-1 (BDX) was prepared using methods that were previously described in the literature (30). Two reaction mixtures were prepared. The first mixture consists of 15 μM Galectin-3 alone. The second mixture consisted of 15 μM Galectin and 30 μM β-Catenin. The two mixtures were incubated overnight at 4 ^{o}C. The next day BDX was added to both mixtures to a concentration of 100 μM. The two mixtures were further incubated overnight at 4 ^{o}C. Equal volumes of 2X SDS-PAGE loading buffer were added to each mixture. After boiling for 15 minutes the mixtures were then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Horse Radish Peroxidase (HRP) Linked Anti-Biotin Antibody Purchased from Cell Signaling^{TM} was diluted 1,000 fold in milk/TBS-T and applied to the membrane for 1 hour. The membrane was washed three time with TBS-T, 15 minutes per wash. Pierce^{TM} ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One^{TM}.

The BDX assay described was also performed to determine whether or not α-Actinin binding to Galectin-3 had any effect upon the ability of DX-52-1 to binding to Galectin-3. The only difference at the beginning was the composition of the two mixtures prepared. The first

mixture consists of 15 μM Galectin-3 alone. The second mixture consisted of 5 μM Galectin and 20 μM α-Actinin.

Pulldown Assays of DX-Galectin-3/β-Catenin Complex by α-Actinin

On the first day a DX binding mixture consisting of 1 μM Galectin-3 and 1 mM DX-52-1 as well as a control mixture of 1 μM Galectin-3 and 1% DMSO were incubated overnight at 4 ^{o}C. On the second day the control and DX binding mixtures were diluted 10X with water. One volume of 5 μM β-Catenin was added to two volumes of the DX binding mixture. 1 volume of 5 μM β-Catenin was added to two volumes of the control mixture. The mixtures were then incubated overnight at 4 ^{o}C. On the third day the mixtures were diluted 10X with water. Next a blank mixture consisting of β-Catenin alone was set to a concentration equaling the β-Catenin concentrations of the control and DX binding mixtures following their dilution with water as previously described. Equal volumes of α-Actinin bearing beads (Preparation described earlier in this section) were added to each mixture. These mixtures were then rotated overnight at 4 ^{o}C. One the fourth day the beads in the binding and blank mixtures were spun down and the supernatants discarded. The beads were washed three times with His Tag binding buffer with final volumes being minimized. 40 μl SDS-PAGE loading buffer is added to each set of beads. After boiling for 15 minutes the mixtures were then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Rabbit anti β-Catenin purchased from Sigma-Aldrich^{TM} was diluted 1,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. The membrane was washed three times with TBS-T, 15 minutes per wash. Goat antirabbit-HRP was diluted 10,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. Once again the membrane was washed three time with TBS-

T, 15 minutes per wash. Pierce™ ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One™. The binding of the Galectin-3/β-Catenin Complex by α-Actinin in each lane is defined as the intensity of the β-Catenin band minus the intensity of the β-Catenin band in the lane of the blank mixture were Galectin-3 was never present. The relative binding of the Galectin-3/β-Catenin Complex in each lane is summarized by the Equation 1.

$$Relative\ Binding = \frac{\beta Cat\ Band\ Intensity - \beta Cat\ Band\ Intensity\ (Blank\ Lane)}{\beta Cat\ Band\ Intensity\ (Control\ Lane) - \beta Cat\ Band\ Intensity\ (Blank\ Lane)}\ x\ 100\%$$

Equation 1: Relative Binding of Galectin-3/β-Catenin Complex to α-Actinin, βCat Band Intensity: Sample involves DX-52-1 bound Galectin-3, Control Lane: Sample involves Galectin-3 without DX-52-1, Blank Lane: Galectin-3 absent from Sample

Results and Discussion

In the course of our work we have sought to find a link between the ability of DX-52-1 to bind to Galectin-3 and its ability to reduce both cell migration and proliferation, both observations made by Kahsai et al. In our earlier work described in Chapter 1 we have observed that the interaction of Galectin-3 and six out ten of the binding partners we have tested were reduced by DX-52-1. These reductions were relatively modest. However if this "six out of ten" ratio holds for the multitude of Galectin-3 binding partners then even modest reductions could yield significant effects on cell function as a whole. We then sought to determine if DX-52-1 has a greater than modest effect on the formation of higher order proteins complexes that contain Galectin-3. We have chosen β-cat/Gal-3/α-act as our test case. The reason for this selection is that both β-Catenin and α-Actinin both are involved in cell migration which we in the course of our work also study.

We first sought to demonstrate though a pull down assay of β-Catenin by α-Actinin, that Galectin-3 does in fact provide a link between β-Catenin and α-Actinin, in order to establish the

importance of Galectin-3 in formation of the β-cat/Gal-3/α-act Complex. Though further pull down assays we observed the effects of DX-52-1 on the formation of this complex and compared them to the effects of DX-52-1 on the binding of Galectin-3 to β-Catenin and α-Actinin individually. Due to the fact that both β-Catenin and α-Actinin are very large compared to Galectin-3 we postulated that each protein likely covers a high percentage of the surface area of Galectin-3 and as such their binding sites are likely on opposite sides of Galectin-3 to make such an arrangement geometrically feasible. Though a series of BDX binding assays we sought to determine whether the DX-52-1 coincided with β-Catenin or α-Actinin. The two aforementioned observation led us to propose the layout of the β-cat/Gal-3/α-act Complex shown in Figure 2-2.

Figure 2-2: Proposed Layout of β-Catenin/Galectin-3/α-Actinin Complex, Proposed location of DX-52-1 binding site relative to β-Catenin is based on the observed ability of that protein to block the binding of DX-52-1 to Galectin-3, α-Actinin demonstrates no such blocking ability and as such is suspected of binding to the opposite side of Galectin-3 relative to the β-Catenin/DX-52-1 binding site due the enormous sizes of α-Actinin and β-Catenin relative to the size of Galectin-3

In the pull down assays of the Galectin-3/β-Catenin Complex by α-Actinin, the results show that binding Galectin-3 to β-Catenin can in fact provide a link between α-Actinin and β-Catenin (Figure 2-3). In the absence of Galectin-3, β-Catenin binding to α-Actinin is reduced to 25.8% ± 3.11% relative to such binding when Galectin-3 and β-Catenin are allowed to form a complex beforehand. Although the results of the pull down assay does show some α-Actinin/β-Catenin binding even the complete absence of Galectin-3, it is quite clear that Galectin-3 does have a very substantial influence on the ability of β-Catenin to form a complex with α-Actinin. So it does stand to reason that β-Catenin, Galectin-3, and α-Actinin do in fact form a three protein complex (β-cat/Gal-3/α-act). The interaction between β-Catenin and α-Actinin is generally described in the literature to take place through α-Catenin (24, 27). There is as of yet no mention of an in vivo interaction between β-Catenin and α-Actinin via Galectin-3 in the literature. However in our attempt to explain the strong cellular effects of DX-52-1 as well as determine a link between the specific binding of DX-52-1 to Galectin-3 and such effects, the β-Cat/Gal-3/α-Act complex has provided a useful test case in that DX-52-1 may have larger effect on the formation of higher order Galectin-3 containing structures than it would on two-protein containing Galectin-3 containing complexes.

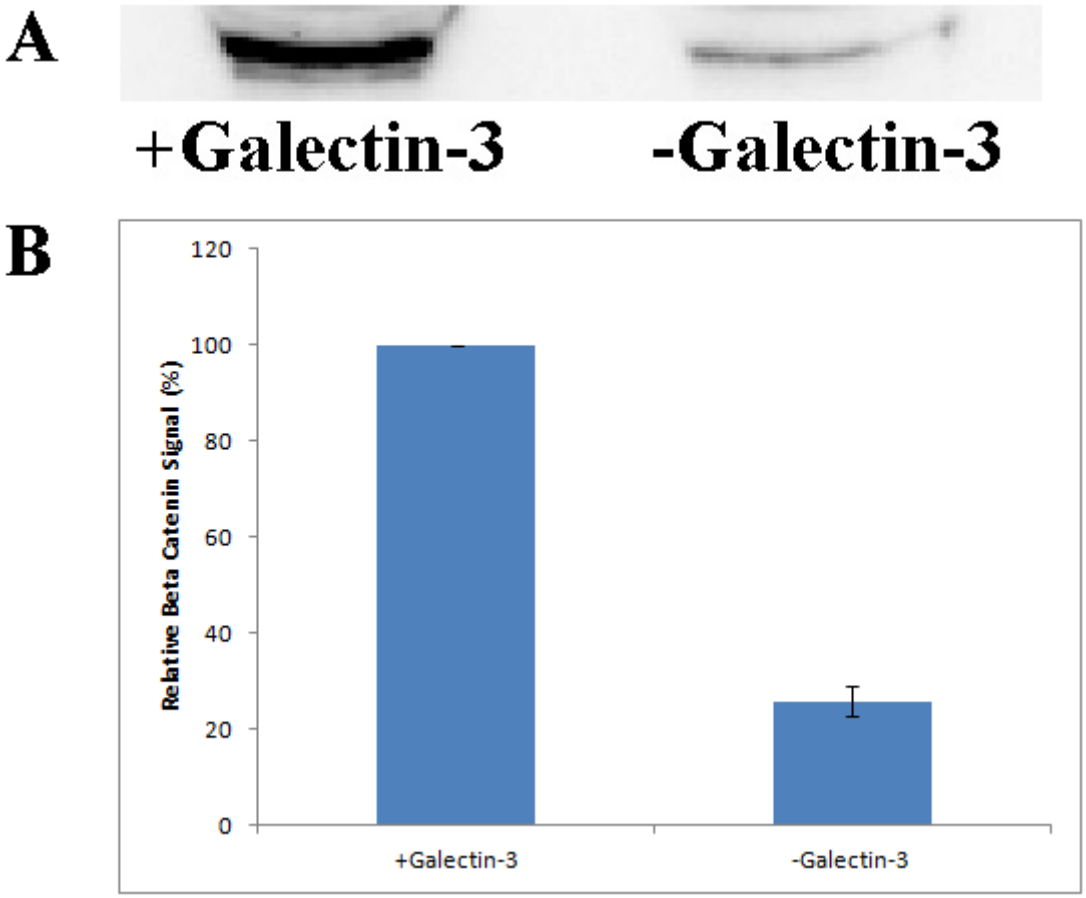

Figure 2-3: Effect of Galectin-3 on the Binding of β-Catenin to α-Actinin

A) Western Blot of Pull Down Assay of β-Catenin by α-Actinin, with Galectin-3 Pre-Bound to β-Catenin (left lane) vs. β-Catenin alone (right lane)
B) Comparison of the Relative Intensity Relative Intensity of β-Catenin Signal. Relative Intensity is defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the +Galectin-3 Control Sample (SD, $n = 3$), The difference in Binding was statistically significant ($p < 0.0001$)

The next thing we have sought to probe was the effect of DX-52-1 upon the binding of Galectin-3 to α-Actinin and β-Catenin individually. The results of the pull down assays we performed have shown that DX-52-1 does not have a statistically significant effect on the binding of Galectin-3 and β-Catenin but does in fact have a statistically significant effect on the binding between Galectin-3 and α-Actinin (Figure 2-4). DX-52-1 seems have reduced the binding of Galectin-3 and β-Catenin to 92.3% ± 8.14%. More significantly DX-52-1 has reduced the binding

between Galectin-3 and α-Actinin to 66.4% ± 11.0%, which is in line with the positive results we have obtained in similar pull down assays with other Galectin-3 binding partners (unpublished results). DX-52-1 has a modest but significant effect on Galectin-3 binding to α-Actinin and little if any effect on Galectin-3 binding to β-Catenin. We had initially predicted that any effect that DX-52-1 has on the formation of the β-cat/Gal-3/α-act complex would be due mostly if not completely to DX-52-1 undermining the binding between Galectin-3 and α-Actinin. Furthermore we had predicted that such a reduction would be roughly the same as the reduction of Galectin-3 binding to α-Actinin. However the results we have obtained had completely contradicted those initial predictions and showed the DX-52-1 has a much more profound effect on the formation of β-cat/Gal-3/α-act complex than it does on the simple Gal-3/α-act Complex.

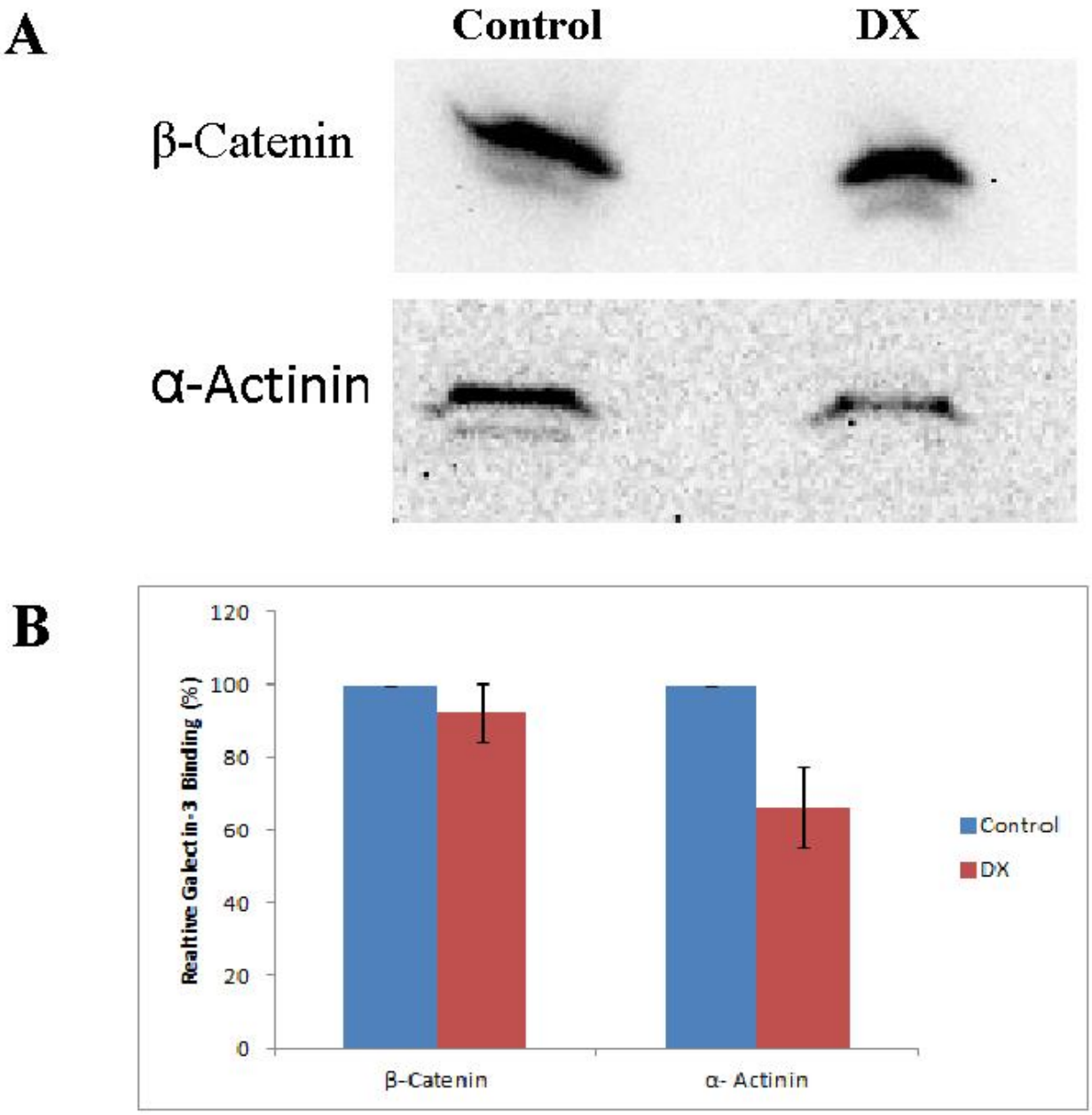

Figure 2-4: Effect of DX-52-1 on the Binding of Galectin-3 to β-Catenin and α-Actinin

A) Western Blots of Pull Down Assays of Galectin-3 by β-Catenin and α-Actinin, Galectin-3 Control (left lanes) and Galectin-3 bound with DX-52-1 (right lanes)
B) Relative Galectin-3 Signal (left lanes [control] vs. right lanes [DX]), Relative Intensity is defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Galectin-3 Control (SD, *n* = 3), DX-52-1 did not show a statistically significant effect in the pull down of Galectin-3 by β-Catenin, DX-52-1 did show a statistically significant effect in the pull down of Galectin-3 by α-Actinin (p = 0.014)

We then proceeded to probe the effect of DX-52-1 on the ability of a β-Catenin/Galectin-3 complex to bind to α-Actinin. Our pull down assay has demonstrated that the ability of α-Actinin to bind to β-Catenin that was in a complex with Galectin-3 bound with DX-52-1 was not significantly greater that its ability to bind β-Catenin in the complete absence of Galectin-3 (Figure 2-5). We have defined in Equation 1, the net signal for the binding of the β-Catenin/Galectin-3 complex to be the signal over and above the signal for β-Catenin binding α-Actinin in the complete absence of Galectin-3. The net signal for the binding of α-Actinin by the β-Catenin/Galectin-3 complex when Galectin-3 is bound with DX-52-1 is 5.70% ± 2.73% of the net signal when the β-Catenin/Galectin-3 complex is free of DX-52-1. This result stood complete in contrast to what we had expected to observe based on the results of the individual pull down assays of Galectin-3 by β-Catenin and α-Actinin. Our previous unpublished results had shown that DX-52-1 had at best only moderate effects on the ability of Galectin-3 to form two-protein complexes. It now seems that DX-52-1 may in fact have the ability to severely undermine the formation of higher order protein complexes involving Galectin-3. Our test case involving β-Cat/Gal-3/α-Act complex demonstrates that this may indeed be a possibility. The possible effect of DX-52-1 on higher order Galectin-3 bearing complexes may serve to partially explain why DX-52-1 has a strong cellular effects and how DX-52-1 binding to Galectin-3 may play a role.

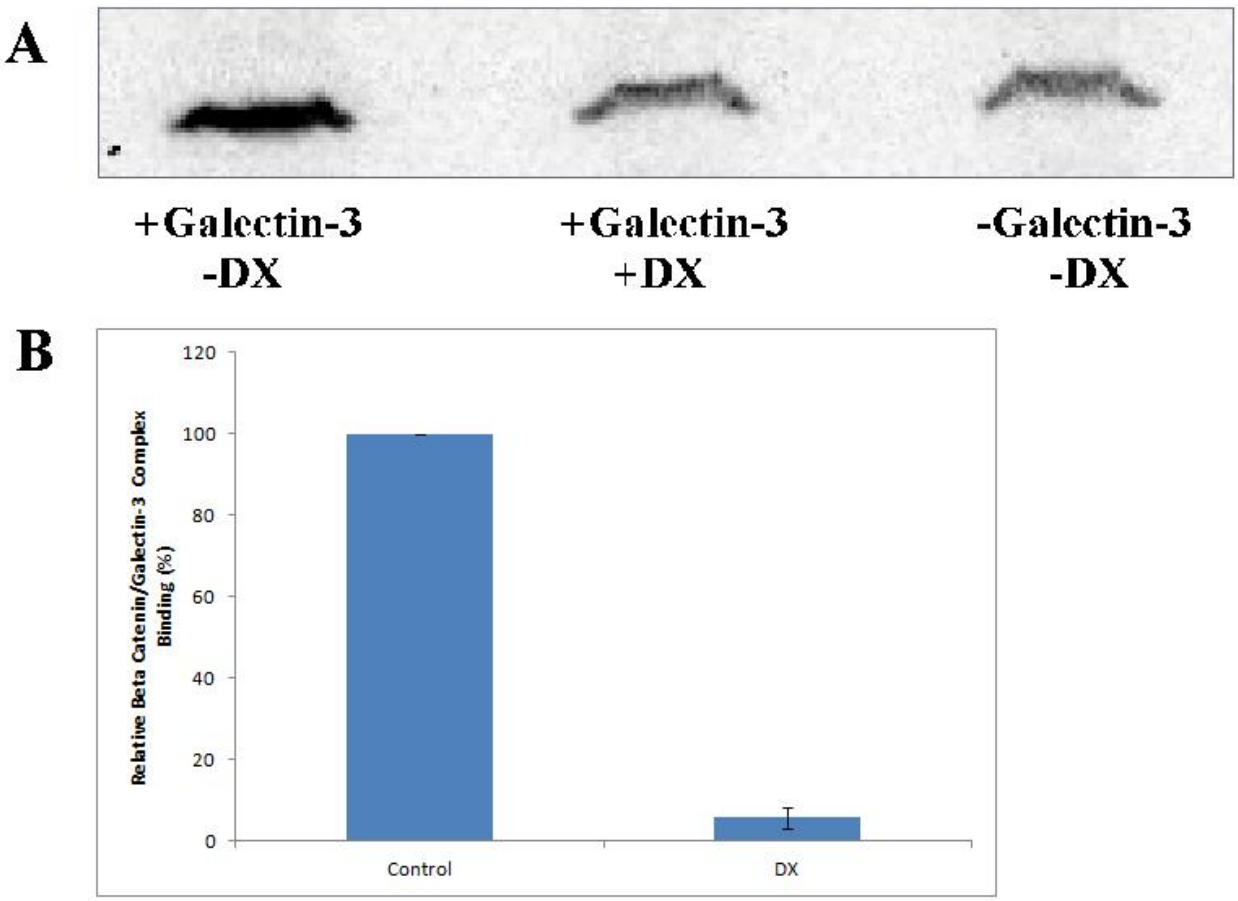

Figure 2-5: Effect of DX-52-1 on the Binding of β-Catenin/Galectin-3 Complex to α-Actinin

A) Western Blot of β-Catenin Pull Down Assay by α-Actinin, β-Catenin Pre-Bound with Galectin-3 (left lane) vs. β-Catenin Pre-Bound with DX Bound Galectin-3 (middle lane) vs. β-Catenin without Galectin-3 (right lane)
B) Relative Net β-Catenin Signal defined as the ratio of the difference between the directly measured signal intensities of the sample and the Blank Sample [-Galectin-3 and –DX] vs. the difference between the directly measured signal intensities of the Control Sample [+Galectin-3 and –DX] (SD, $n = 3$), Binding DX-52-1 to Galectin-3 showed a statistically significant effect in the pull down of β-Catenin/Galectin-3 ($p < 0.0001$), β-Catenin that was pre-bound with DX-52-1 bound Galectin-3 gave a signal intensity only marginally higher than β-Catenin in the complete absence of Galectin-3 (+Galectin-3/+DX vs. –Galectin-3/-DX)

In addition to probing the effect of DX-52-1 on the binding of β-Catenin and α-Actinin to Galectin-3, we have also probed the effect of β-Catenin and α-Actinin on the ability of DX-52-1 to bind to Galectin-3. In doing so we have been able to make a rough estimation of where on Galectin-3 DX-52-1 binds in relation to β-Catenin and α-Actinin. Our BDX binding assay on the

effect of β-Catenin upon the binding of DX-52-1 to Galectin-3 show such binding to be all but eliminated (Figure 2-6). When bound to β-Catenin, the binding of BDX by Galectin-3 is reduced to 2.54% ± 1.23%. The near completeness of this effect suggests that DX-52-1 binding site of Galectin-3 very likely coincides with the binding site of β-Catenin.

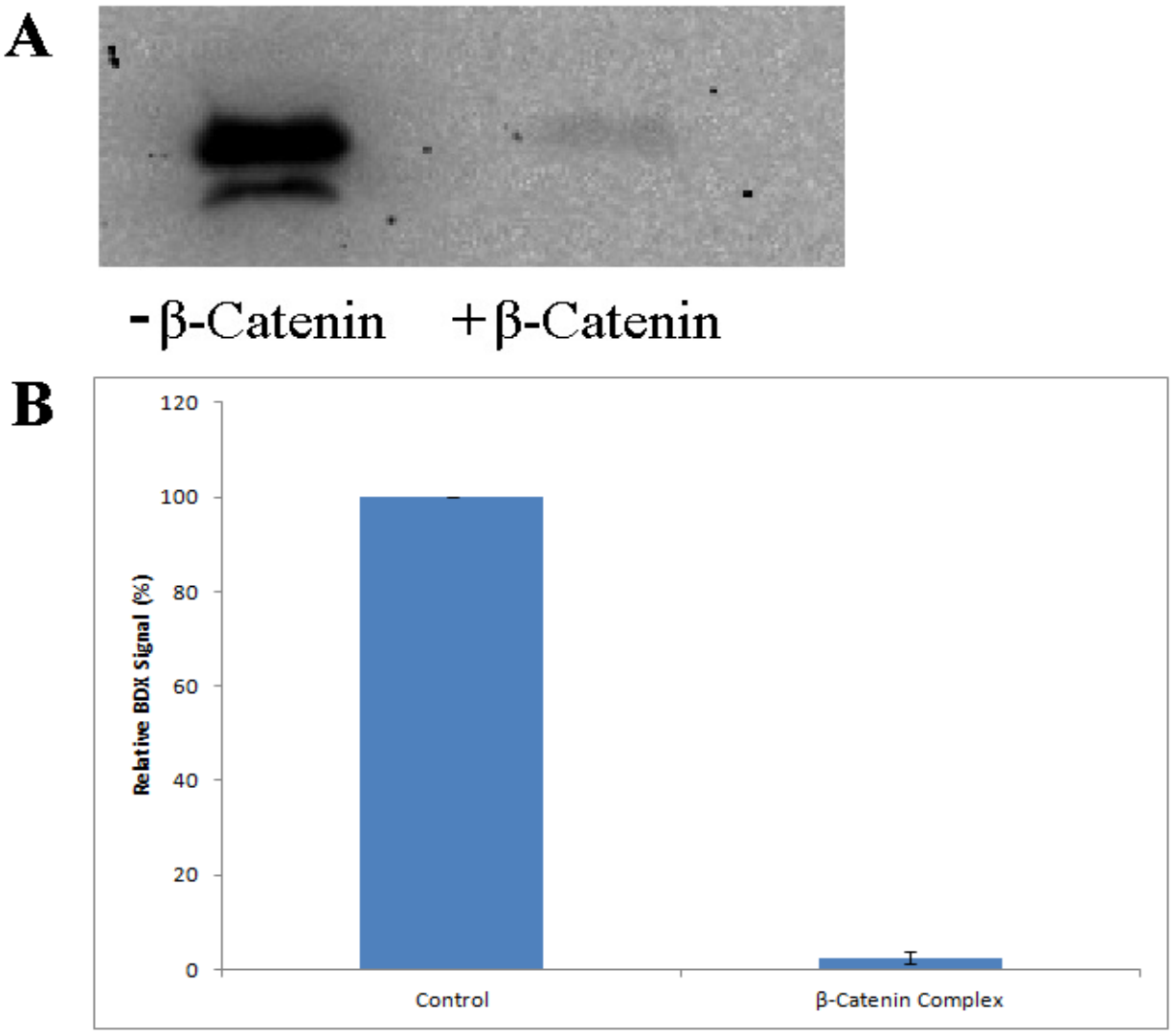

Figure 2-6: Effect of β-Catenin on the binding of DX-52-1 on Galectin-3

A) Western Blot of BDX Assay of Galectin-3, Galectin-3 Control (left lane) vs. Galectin-3 Pre-bound with β-Catenin (right lane)
B) Relative BDX Signal defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Galectin-3 Control Sample (SD, *n*

= 3), β-Catenin reduced the extent of BDX to Galectin-3 by a statically significant amount ($p < 0.0001$)

Next we proceeded to determine the effect of α-Actinin on the ability of DX-52-1 to bind Galectin-3. The BDX binding assay showed there was no reduction of BDX binding to Galectin-3 by α-Actinin (Figure 2-7). When in a complex with α-Actinin Galectin-3 binds DX-52-1 96.6% ± 1.41% as much it does when α-Actinin is completely absent, which suggests that the binding site of α-Actinin does not in any way overlap with the binding site of DX-52-1 unlike β-Catenin. Serendipitously our BDX Assay had also shown that DX-52-1 also binds to α-Actinin. The literature had previously shown that in the micromolar range DX-52-1 binds to Galectin-3 and Radixin (30, 31). It seems that α-Actinin may also be placed upon that list. Due to the fact that β-Catenin all but eliminates DX-52-1 binding to Galectin-3 while α-Actinin has practically no effect at all, it can be reasonably concluded that DX-52-1 binding to Galectin-3 on the roughly same side as β-Catenin while α-Actinin binds on a completely different side of Galectin. If that conclusion truly sound it might be further concluded that the effect of DX-52-1 on the binding of Galectin-3 to α-Actinin is likely due to a conformational shift brought about by DX-52-1 since its binding site the possibility of steric interference is excluded by the aforementioned conclusion, as the DX-52-1 binding site does not overlap with α-Actinin.

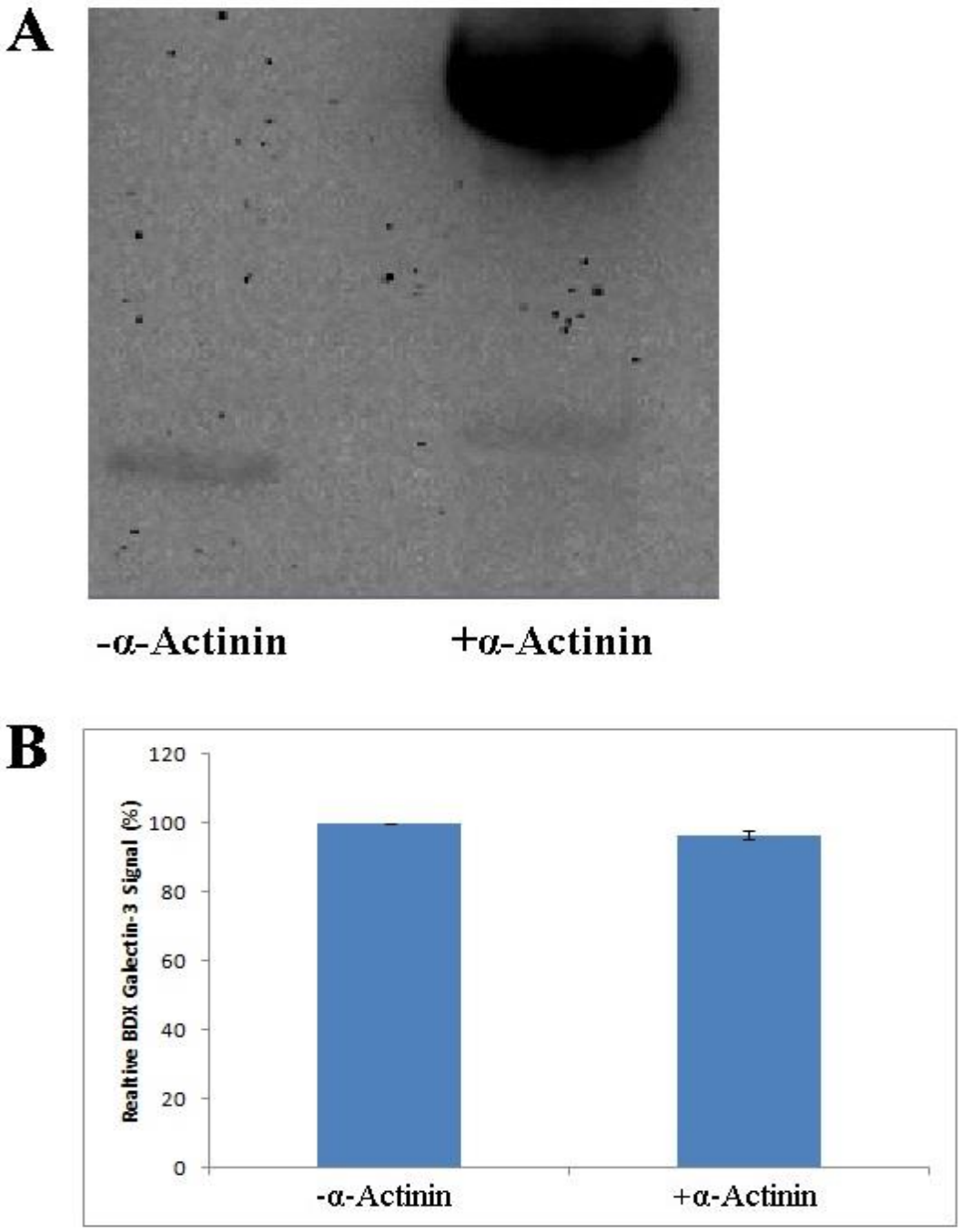

Figure 2-7: Effect of α-Actinin on the binding of DX-52-1 on Galectin-3

A) Western Blot of BDX Assay on Galectin-3, Galectin-3 Control (left lane) vs. Galectin-3 Pre-bound with α-Actinin (right lane)
B) Relative BDX Signal (left lane vs. right lane) defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Galectin-3 Control Sample [-α-Actinin] (SD, $n = 3$), α-Actinin did not reduce the binding of BDX to Galectin-3 by any statistically significant amount, Also of note it turns out α-Actinin can itself bind BDX.

In this work we have shown that Galectin-3 forms a three protein complex with β-Catenin and α-Actinin. We have further demonstrated DX-52-1 has a much stronger effect on the formation of the β-Cat/Gal-3/α-Act complex than it does on the formation of two-protein complexes of Galectin-3 with either β-Catenin or α-Actinin individually thus showing the possibility of DX-52-1 having a stronger effect on the formation of higher order complexes. This suggests that in the development of DX-52-1 and similar small molecules as medical treatment may make necessary the exploration of its effect on Galectin-3 protein complexes with two or more additional proteins. Our deduction of the general location of the DX-52-1 binding site is somewhat fortuitous considering difficulty of crystallizing Galectin-3 for x-ray crystallography, which make a direct determination of the binding site difficult (4). We are confident that further study of Galectin-3 with open up several more avenues of research.

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Chapter 3

Mapping of DX-52-1 Binding Peptide on Galectin-3

Introduction

Throughout the literature Galectin-3 has been shown to play a role in a number of cellular and pathogenic processes (1-8). The data provided in the previous two chapters have shown the quinocarcin analog DX-52-1 has a demonstrable effect on the ability of Galectin-3 to interact with its binding partners. Furthermore DX-52-1 has been shown to exhibit inhibitory properties on both cell proliferation and migration. The link between these two properties and Galectin-3 was established by Kahsai et al and is further supported by the results that will be described and discussed in Chapter 4 (9). In order to properly understand the mechanism by which the interaction between DX-52-1 and Galectin-3 exerts its effects on binding partner interaction, cell proliferation and cell migration, it is important to ascertain the exact position on Galectin-3 to which DX-52-1 binds.

Normally this determination would be accomplished by running DX-52-1 bound Galectin-3 alongside control Galectin-3 on a polyacrylamide gel followed an in-gel tryptic digestion for submission to LC-MS/MS analysis. However in the case of Galectin-3 this has proven problematic. In fact a comparison of the mass spectra of even undigested DX-52-1 bound Galectin-3 and control Galectin-3 show absolutely no mass shift due to DX-52-1 (Figure 3-1). It is clear that at some point in either sample preparation for MS or the MS process itself the bound DX-52-1 is somehow lost.

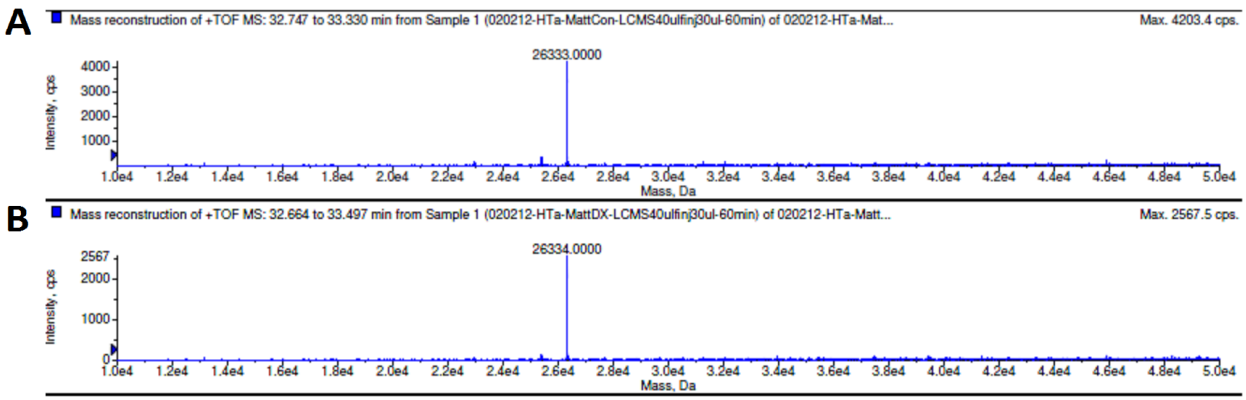


Figure 3-1: MALDI-TOF ES+ data shows (A) m/z of 26.3 kDGalectin-3 untreated and (B) m/z of 26.3 kD DX-52-1 treated Galectin-3

Alanine scanning via site directed mutagenesis was selected as an alternative strategy to mass spectrometry. Alanine scanning essentially involves creating a series of mutant proteins, each of have a residue of interest substituted with alanine for the purpose of determining those residues' impact on the function of a protein (10-14). Assuming the mechanism proposed by Kashai et al is in fact correct then any Galectin-3 residue with a nucleophilic side chain is a possible candidate. Twenty seven such candidates were identified on CRD alone. The project described in this chapter concerns both the determination on the cause of the loss of DX-52-1 binding with Galectin-3 and the development of a method to reduce the number of candidate residues for alanine screening.

The DX-52-1 binding residue can be localized in terms of protein sequence by isolating the tryptic fragment that contains that residue (Figure 3-2). It is worth noting that the cleavage sites for tryptic digestion of Galectin-3 are all to be found in the second half of the protein, essentially the CRD portion (Figure 3-3). If it had turned out that DX-52-1 binds to the ND section, it would have been necessary to use a different protease. It was hypothesized that the reason for DX-52-1 being lost by Galectin-3 was due to the final step in the MS sample preparation which involves acidification. This forms the cornerstone of the proposed DX binding peptide isolation method in this chapter. The first step is binding BDX to Galectin-3. The second step is binding the

BDX/Galectin-3 to streptavidin agarose beads. Any protein that fails to bind to the beads is washed away. The third step involves the beads bearing Galectin-3 being subjected to trypsin proteolysis. Any tryptic fragment that is not direct bound to the BDX would break away as a result. In the fourth step the beads would then be treated with formic acid and presumably the bond between BDX and the Galectin-3 peptide bound to it would be broken. The beads would be removed by centrifugation and the peptide containing the DX-52-1 binding residue should remain in the supernatant. The sequences of this peptide would be determined by MS/MS. This would reduce the list of DX-52-1 binding residue candidates significantly.

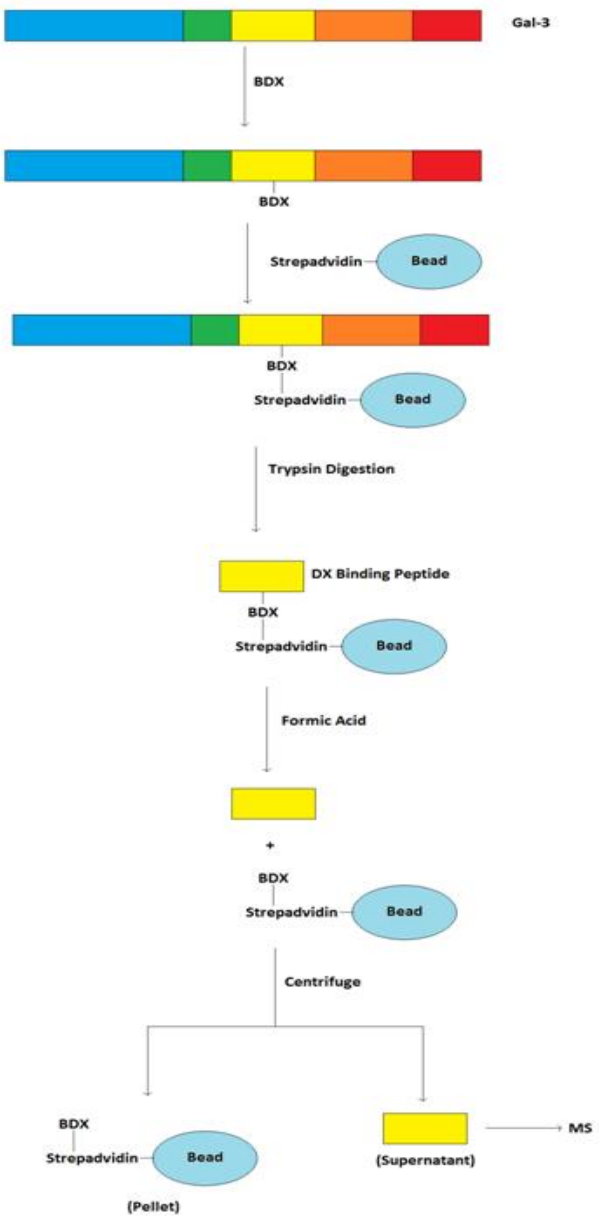


Figure 3-2: Scheme of DX-52-1 Binding Peptide Isolation Protocol. Streptavidin beads bind the biotin component of BDX bound Galectin-3. After Trypsin digestion only the fragment directly attached to BDX remains linked to the Streptavidin bead. Formic acid treatment breaks the bond between that and BDX. The former BDX bound peptide is then sequenced via MS/MS.

Materials and Methods

Preparation of Galectin-3 and CRD

Galectin-3 was prepared for this project using the same protocol that was described in the Materials and Methods section of Chapter 1. CRD, which was expressed using a pGEX-6P-1 plasmid, was expressed using a protocol similar to that which was employed for Galectin-3 but with a few variations. The first variation is the time in which the expressing cells were induced with IPTG was 3 hours instead of the usual 5 hours. The second variation is that the protease PreScission™ (Purchased from GE Healthcare) was used to cleave CRD from its GST tag instead of Thrombin.

BDX Assay for CRD

On the first day a reaction mixture consisting of 100 µM CRD and 1 mM BDX was prepared. The reaction mixture was then incubated at 4 ^{o}C overnight. On the second day equal volumes of 2X SDS-PAGE loading buffer was added to the mixture. After boiling for 15 minutes the mixture was then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Horse Radish Peroxidase (HRP) Linked Anti-Biotin Antibody Purchased from Cell Signaling™, was diluted 1,000 fold in milk/TBS-T and applied to the membrane for 1 hour. The membrane was then washed three time with TBS-T, 15 minutes per wash. Pierce™ ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One™.

BDX/Acid Assay for Galectin-3

On the first day a reaction mixture consisting of 25 µM Galectin-3 and 200 µM BDX was prepared. The reaction mixture was then incubated at 4 ^{o}C overnight. On the second day the reaction mixture was divided into two samples. Formic acid to a concentration of 7% was added to one of the samples. An equivalent volume of water was then added to the other sample. The two samples were then incubated overnight at 4 ^{o}C. On the third day the two samples were subject to the same western blot procedure that was employ for CRD as described in the previous paragraph.

Catch and Release Test for Galectin-3

On the first day a reaction mixture consisting of 25 µM Galectin-3 and 200 µM BDX was prepared. This reaction mixture was then incubated at 4 ^{o}C overnight. On the second day an equal volume of streptavidin-agarose beads were added to the reaction mixture. The reaction mixture was rotated overnight at 4 ^{o}C. On the third day the beads were centrifuged. The supernatant was designated "Supernatant 1" and stored for later use. The beads were then washed three times with water and the final volume was minimized. The beads were then re-suspended in 7% formic acid. The beads were then rotated overnight at 4 ^{o}C. On the fourth day the beads were centrifuged and the supernatant removed was designated "Supernatant 2" Equal volumes of SDS-PAGE loading buffer were added to Supernatant 1 and Supernatant 2. The beads were mixed with 20 µl of SDS-PAGE loading buffer. After boiling for 15 minutes the mixtures were then run on a 12% polyacrylamide gel at 250 volts for 35 minutes before being transferred to PVDF membrane at 200 mA for 1 hour. The membrane was blocked by a 5% milk/TBS-T solution for 1 hour. Mouse antigalectin-3 purchased from Santa Cruz Biotechnology^{TM} was diluted 1,000 fold in 5% milk/TBS-T and applied to the membrane for 1 hour. The membrane was washed three times with TBS-T, 15 minutes per wash. Goat antimouse-HRP was diluted 10,000 fold in 5% milk/TBS-T

and applied to the membrane for 1 hour. Once again the membrane was washed three times with TBS-T, 15 minutes per wash. Pierce™ ECL Western Blotting Reagent was applied to the membrane for five minutes. The bands were visualized and then quantified by Bio-Rad Quantity One™.

DX-52-1 Binding Peptide Isolation

On the first day three identical reaction mixtures consisting of 25 µM Galectin-3 and 200 µM BDX were prepared. These reaction mixtures were then incubated at 4 ºC overnight. On the second day equal volumes of streptavidin beads were added to each reaction mixture. The reaction mixtures were rotated overnight at 4 ºC. On the third day the mixtures were centrifuged and the beads were retained. Each set of beads was re-suspended in 4M-urea/25 mM ammonium bicarbonate and rotated at room temperature for 15 minutes. The beads were centrifuged and the supernatant was discarded. The two aforementioned steps were repeated twice and the final volumes were all minimized. The beads were re-suspended in 10 mM DTT/25 mM ammonium bicarbonate and rotated at 37 ºC for 45 minutes. The beads were then centrifuged and the supernatant was discarded. The beads were washed with 4M urea/25 mM ammonium bicarbonate as previously described. The beads were then re-suspended in 20 mM Iodacetimide and rotated at room temperature in the dark for 1 hr. The beads were once more washed with 4M urea/25 mM ammonium bicarbonate as previously described. The beads were then resuspened in 12.5 ng/µl Trypsin/40 mM ammonium bicarbonate. The beads were then rotated overnight in the dark at 37 ºC. On the fourth day the beads were centrifuged and the supernatant was discarded, the beads were washed with water three times and the final volumes were all minimized. Each set of beads was re-suspended in 7% formic acid. The beads were then rotated overnight at 4 ºC. On the fifth day the beads were spun down and the supernatants were retained. The three supernatants were

pooled together and the solvent was removed by spin vac. The residue was then re-suspended in 20 µl 0.1% formic acid. The sample was submitted for LC-MS/MS analysis. The amino acid sequence derived from the mass spectrometry data was then compared to the sequences of the predicted tryptic fragments for CRD (Figure 3-3).

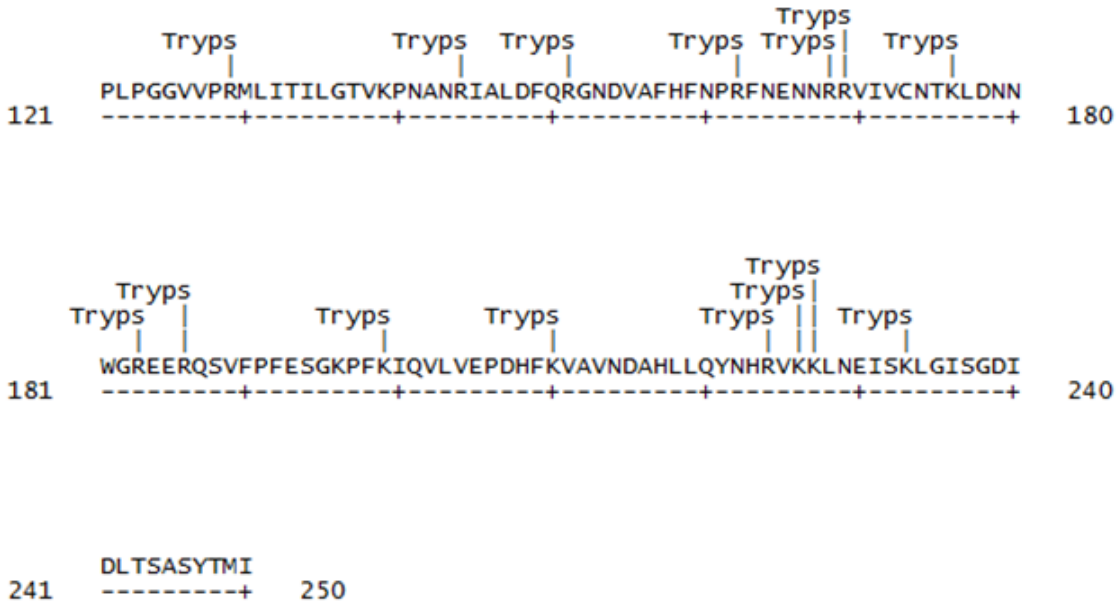


Figure 3-3: Trypsin Cleavage Map of Galectin-3 CRD Domain

Results and Discussion

It was determined by BDX assay that DX-52-1 binds to the CRD region of Galectin-3 (Figure 3-4). It is worth noting that the amount of both BDX and protein required to generate a substantial signal is higher than what is required in the case of full length Galectin-3. The BDX assay for CRD involved 100 µM CRD and 1 mM BDX. In the case of full length Galectin-3 the BDX assay will yield a detectable signal even when the concentration of Galectin-3 and BDX are as low as 12.5 µM and 25 µM respectively (Fenteany Group Unpublished Results). The ND region however showed no BDX binding signal even at concentrations higher than those employed for CRD. It can therefore be reasonably concluded that DX-52-1 binds to the CRD region of Galectin-

3. It can be further concluded that although the ND region itself does not bind DX-52-1 it does play a role in the binding of DX-52-1 to Galectin-3. The CRD of Galectin-3 has a total of 27 amino acids that have nucleophilic side chains. These residues include Y118, T133, T137, K139, H158, C173, T175, K176, S118, S194, K196, K199, H208, K210, H217, Y221, H223, K226, K227, S232, K233, S237, T243, S244, S246, Y247, and T248.

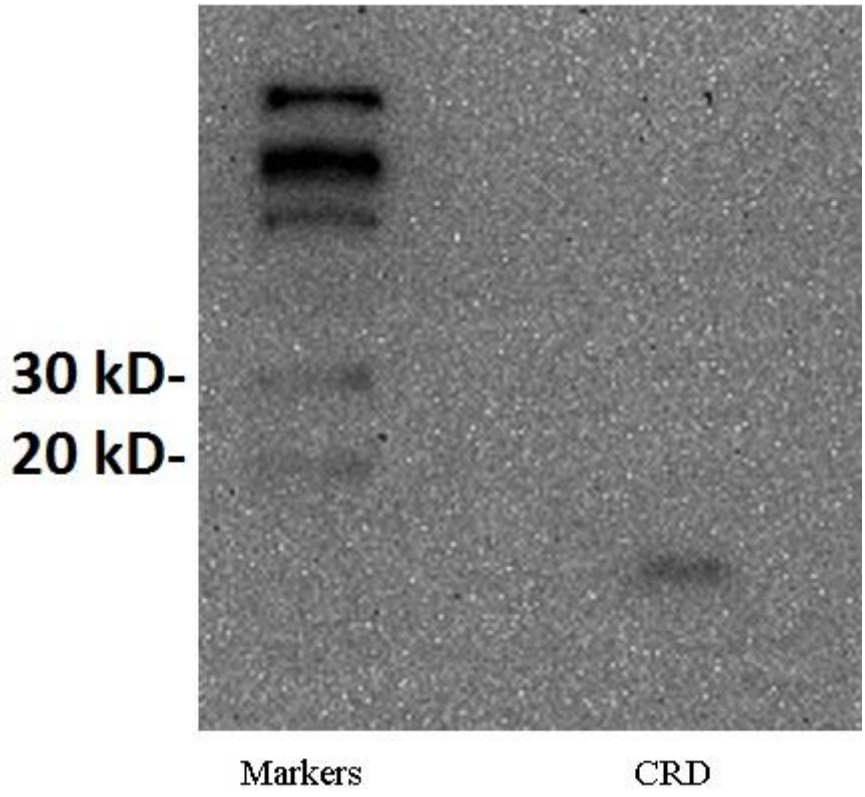


Figure 3-4: BDX Assay of CRD, 100 µM Galectin-3 CRD was incubated with 1 mM BDX overnight. This mixture was subjected to a western blot. The membrane was probed using antibitin-HRP antibody. Result indicates CRD domain of Galectin-3 binds BDX. Assay was performed on N-terminal Domain of Galectin-3. Those results (not shown) were negative.

Formic Acid treatment of BDX bound Galectin-3 results in a near total loss of BDX signal (Figure 3-5). The result implies that acidification causes the BDX and presumably DX-52-1 to break away from Galectin-3. This effect was observed when the formic acid concentration was as little as 7%. It is also noteworthy that a similar effect was observed with BDX bound Radixin, which suggests that the observed effect is due to DX-52-1 alone (Fenteany group Unpublished

Results). The protocol for MS sample preparation of DX-52-1 bound Galectin-3 has formic acid concentrations going as high as 50%. Although this formic acid induced loss of DX-52-1 poses an inconvenience it also provided a way to shorten the list of candidate Galectin-3 residues that would have to be tested through alanine scanning.

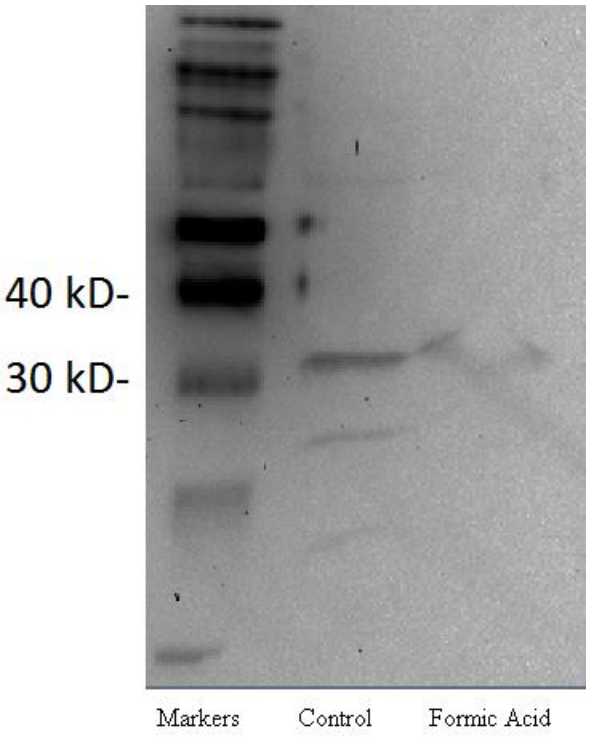


Figure 3-5: Effect of Formic Acid Treatment of DX-52-1 Bound Galectin-3. 25 µM Galectin-3 + 200 µM BDX reaction mixture was incubated overnight and split into two samples. Formic acid was added to the "Formic Acid" Sample to a concentration of 7%. The Formic Acid Sample and Control Samples were subjected to a western blot and the membrane was probed with antibiotin-HRP. Biotin signal is severely reduced in the Formic Acid Sample lane.

In the course of developing the DX-52-1 binding peptide isolation protocol proposed in this chapter a "Catch and Release Test" was performed to verify that formic acid does in fact break the bound between Galectin-3 and DX-52-1. The western blot result obtained at the end of the experiment shows that Galectin-3 was liberated from the streptavidin bead it was bound to through BDX (Figure 3-6). The 7% formic acid used is nowhere near sufficient to break the bound between

streptavidin and the biotin of BDX. Due to this result it was concluded that formic acid should be able to successfully free the DX-52-1 binding peptide from streptavidin beads as well.

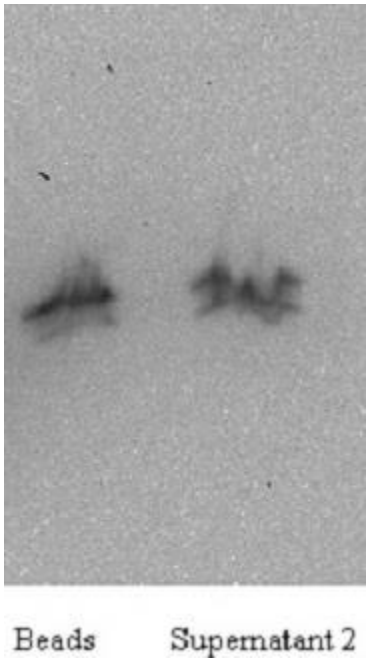
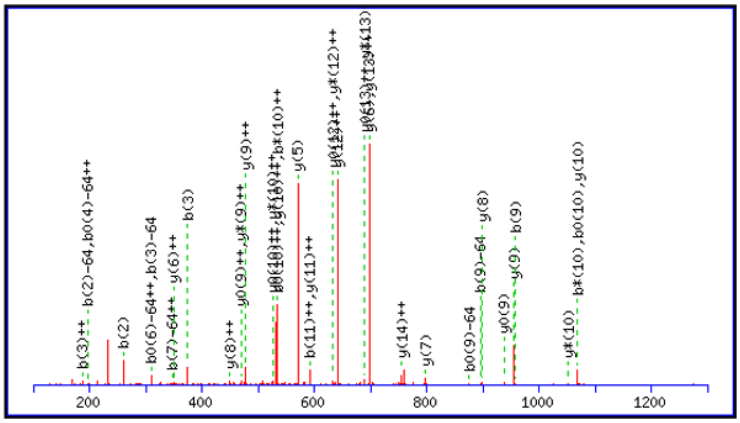


Figure 3-6: Catch and Release Test of Galectin-3. BDX bound Galectin-3 was captured by suspending Streptavidin beads to a reaction mixture of 25 µM Galectin-3 and 200 µM BDX. The beads were spun down by centrifugation. The beads were then suspended in 7% formic acid. "Supernatant 2" is the supernatant of that suspension. Sample of Supernatant 2 and Streptavidin beads were subjected to a western blot and probed with mouse antigalectin-3 primary antibody and antimouse-HRP secondary antibody.

The mass spectrometry results obtained by the University of Connecticut Chemistry Department Mass Spectrometry Facility concerning the sample obtained through the DX-52-1 binding peptide isolation protocol showed the detection of a single peptide with a mass of 1641.00 Daltons. The sequence as determined by MS/MS was MLITILGTVKPNANR [Residues 130-144] (Figure 3-7). This peptide sequence is consistent with the peptides found in the tryptic cleavage map of CRD (Figure 3-3). This amino acid sequence has a total of three of the candidate amino acids out the previously mentioned twenty-seven (T133, T137, and K139). This should make the alanine scanning project an easier undertaking than it might otherwise be.



Monoisotopic mass of neutral peptide Mr(calc): 1655.9392
Variable modifications:
M1 : Oxidation (M), with neutral losses 0.0000(shown in table), 63.9983
Ions Score: 52 **Expect:** 0.15
Matches : 40/190 fragment ions using 41 most intense peaks (help)

#	b	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	148.0427	74.5250					M							15
2	261.1267	131.0670					L	1509.9111	755.4592	1492.8846	746.9459	1491.9006	746.4539	14
3	374.2108	187.6090					I	1396.8271	698.9172	1379.8005	690.4039	1378.8165	689.9119	13
4	475.2585	238.1329			457.2479	229.1276	T	1283.7430	642.3751	1266.7165	633.8619	1265.7324	633.3699	12
5	588.3425	294.6749			570.3320	285.6696	I	1182.6953	591.8513	1165.6688	583.3380	1164.6848	582.8460	11
6	701.4266	351.2169			683.4160	342.2117	L	1069.6113	535.3093	1052.5847	526.7960	1051.6007	526.3040	10
7	758.4481	379.7277			740.4375	370.7224	G	956.5272	478.7672	939.5007	470.2540	938.5166	469.7620	9
8	859.4958	430.2515			841.4852	421.2462	T	899.5057	450.2565	882.4792	441.7432	881.4952	441.2512	8
9	958.5642	479.7857			940.5536	470.7804	V	798.4581	399.7327	781.4315	391.2194			7
10	1086.6591	543.8332	1069.6326	535.3199	1068.6486	534.8279	K	699.3896	350.1985	682.3631	341.6852			6
11	1183.7119	592.3596	1166.6853	583.8463	1165.7013	583.3543	P	571.2947	286.1510	554.2681	277.6377			5
12	1297.7548	649.3810	1280.7283	640.8678	1279.7443	640.3758	N	474.2419	237.6246	457.2154	229.1113			4
13	1368.7919	684.8996	1351.7654	676.3863	1350.7814	675.8943	A	360.1990	180.6031	343.1724	172.0899			3
14	1482.8349	741.9211	1465.8083	733.4078	1464.8243	732.9158	N	289.1619	145.0846	272.1353	136.5713			2
15							R	175.1190	88.0631	158.0924	79.5498			1

Figure 3-7: ESI-MS/MS spectrum of DX-52-1/Galectin-3 Binding Petide MLITILGTVKPNAN (residues 130-144, m/z = 1656 Da), Sequence determined via differences in the m/z of molecular ion fragments of MLITILGTVKPNAN separated by the second mass analyzer

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Chapter 4

Structure-Activity Relationship Study of DX-52-1

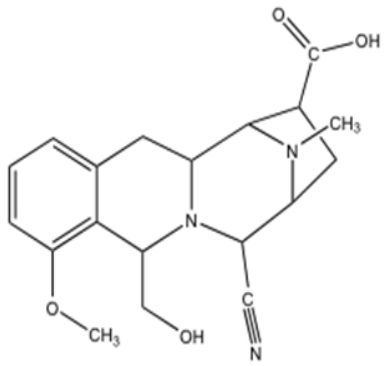
Introduction

Cell motility is a fundamental biological process, that is integral to key processes in mammalian organisms, such as embryonic development, tissue repair, immune response, inflammation and angiogenesis (1, 2) Furthermore cell migration also plays important role in cancer cell invasion and metastasis (3, 4). The rates of cell motility along with cell proliferation are both very important factors in determining the progression of cancers so it stands to reason that any small molecule that can inhibit one or both of these processes might form the basis for an anti-cancer treatment. The small molecule might accomplish this task by binding to a protein that is involved in the aforementioned processes and affect its interaction with other proteins involved in such processes. Galectin-3 which is involved in a great number of cellular functions including proliferation and motility is such protein (5-10).

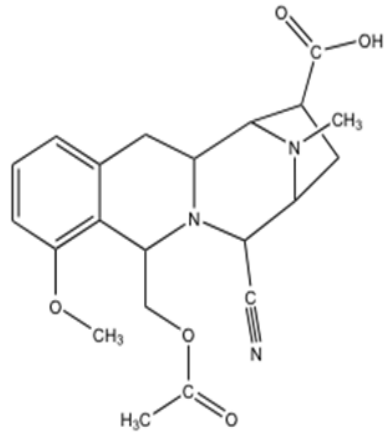
The small molecule studied for the purpose of this project is DX-52-1, (Figure 1-1) an analog of quinocarcin that binds Galectin-3, has been demonstrated to be an inhibitor of cell migration, a key part of tumor cell metastasis (11-13). Furthermore DX-52-1 has also been shown to inhibit cell proliferation to varying extents in a number of mammalian cancer cell lines (Fenteany Group Unpublished Results). DX-52-1 has also been shown to bind Radixin, member of the Ezrin-Radixin-Moesin (ERM) family of proteins (13, 14). The ERM family plays a role in the structure of the cell cytoskeleton and may also play a role in cancer progression (13, 14). Radixin was also shown to play a role in cell migration via the counteracting effects of Radixin over-expression upon the anti-migratory effects of DX-52-1 and furthermore DX-52-1 has been

shown to be a potent inhibitor of the protein-protein interaction between Radixin and its binding partners (13). It is for these reasons that Radixin will be studied alongside Galectin-3.

The objective of this project is to determine the impact of modifying certain functional groups of the DX-52-1 molecule (Figure 4-1). These functional group modifications include the esterification of the hydroxyl group with an acetate (BO1-95), the placement of a bromine atom on the phenyl ring in the para position to the methoxy group (BO2-7), both of the aforementioned modifications on the same molecule (BO1-180), the removal of the nitrile group (1X-108), the substitution of the nitrile group with a ethynyl-TMS group (1X-140), and the replacement of the carboxyl group with a methyl hydroxyl group (1X-141). The impacts that were examined include the Galectin-3 and Radixin binding properties of the aforementioned DX-52-1 derivatives vs. the parent compound as well as the anti-migratory and anti-proliferation effects of the derivatives vs. the parent compound.



DX-52-1



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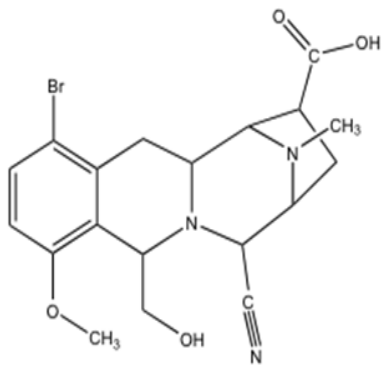

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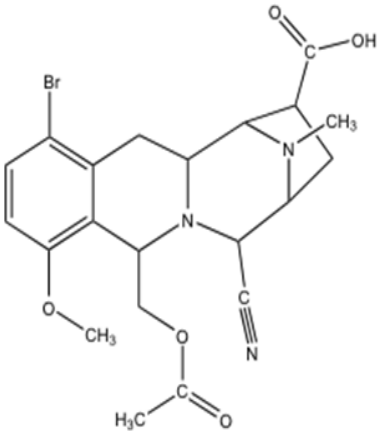

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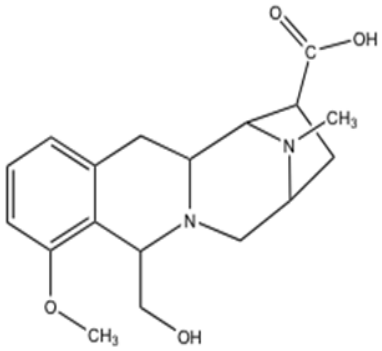
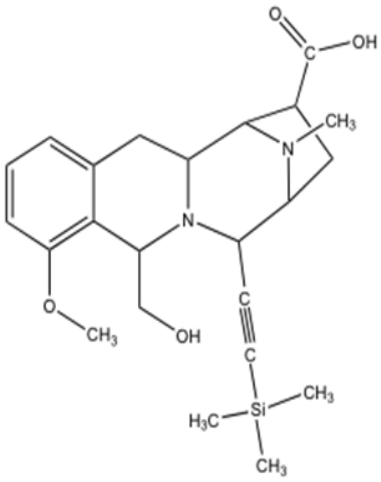
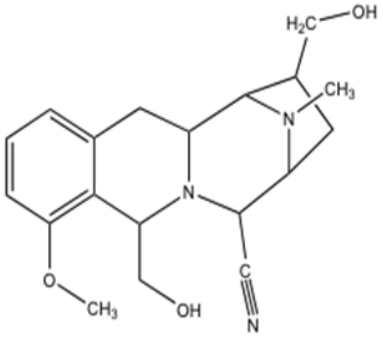


1X-108



1X-140



1X-141

Figure 4-1: Structures of DX-52-1 and its Assorted Derivatives Prepared by Nicholas Eddy

One particular cancer cell line was chosen for these structure-activity relationship (SAR) studies. That cell line is MDA-MB-435, which is derived from malignant breast tumors (15). The rationale for this decision was that DX-52-1 had demonstrated the strongest overall anti-proliferation effects upon MDA-MB-435 in terms of dose response as compared to other cell lines tested (Fenteany Group Unpublished Results). Furthermore MDA-MB-435 also showed a great deal of sensitivity to DX-52-1 in terms of cell migration with regard to dose response (Fenteany Group Unpublished Results). It was reasoned if any of the functional group modifications had a negative impact on the anti-migratory and/or the anti-proliferation effect of DX-52-1 then those effects should be more pronounced in the proliferation and migration assays performed on MDA-MB-435 than any of the other cell lines that were studied.

Materials and Methods

Galectin-3, Radixin, BDX, and DX-52-1 Analogs

The Galectin-3 used in the experiments described in this chapter was prepared used the protocol described in the Materials and Methods section in Chapter 1. The Radixin used was obtained from frozen aliquots stored at -80^{o}C. These aliquots were prepared at an earlier date by former Fenteany Research Group Member Alem Kahsai. The BDX and DX-52-1 analogs were synthesized by Fenteany Research Group Member Nicholas Eddy.

DX-52-1 Analog Competition Assays for Galectin-3 and Radixin

On the first day five reaction mixtures were prepared for Galectin-3. All reaction mixtures contained 20µM BDX and 1.5 µM Galectin-3. The DX Mixture contained 500 µM DX-52-1. The BO1-95, BO1-180, BO2-7, 1X-108, 1X-140, and 1X-141 mixtures each contained 500 µM of the corresponding DX-52-1 analog. The control mixture contained neither DX-52-1 nor any of its

analogs. The reaction mixtures were incubated at 4 ºC overnight. On the second day the reaction mixtures were run on 12% SDS-PAGE gel at 250 V for 35 minutes before being transfer to a PVDF membrane at 200 mA. The membrane was blocked with a 5% milk/TBS-T solution for 1 hour. Antibiotin-HRP antibody was applied to the membrane for 1 hour. The membrane was washed three times with TBS-T, 15 minutes per wash. Pierce ECL Western Blotting Reagent was then applied to the membrane for 5 min. Band intensity was quantified with Bio-Rad Quantity One software. In the case of Radixin the protocol is similar with 1.2 µM Radixin being substituted for Galectin-3.

The relative competition of each DX-52-1 analog was defined by its ability to reduce the intensity of the BDX band relative to the ability of DX-52-1 to do the same. The relative percent competition was computed using the Normalized Relative Competition Equation (Equation 2). This equation is defined in such a way that if the DX-52-1 analog band intensity is equal to the band intensity in the control lane, which would indicate no competition with BDX then the relative percent competition works out to 0%. If DX-52-1 analog band intensity is equal to the band intensity in the DX lane, indicating BDX completion equal to DX-52-1 then the relative percent competition works out to 100%.

$$\%Relative\ Competition\ to\ DX = \frac{\frac{DX\ Band}{Analog\ Band} - \frac{DX\ Band}{Control\ Band}}{1 - \frac{DX\ Band}{Control\ Band}} * 100\%$$

Equation 2: Normalized Relative Competition Equation. “DX Band” refers to BDX western blot signal intensity of sample with DX-52-1 in competition with BDX. “Analog Band” refers to BDX western blot signal intensity of sample with a DX-52-1 derivative in competition with BDX. “Control Band” refers to BDX western blot signal intensity of sample with neither DX-52-1 nor any of its derivatives in competition with BDX.

Cell Cultures

MDA-MB-435 cells were cultured in a growth medium containing D-MEM (Dulbecco's Modified Eagle Medium) and 10% FBS (Fetal Bovine Serum) with 5% CO_2 in a humidified tissue culture incubator. Early passages of cells cultured from frozen stock cultures were used in all experiments. Main cultures were grown in 75-cm^2 tissue culture flasks with medium changed every two days. When the cultures reached 90% confluence, the cells were gently washed twice with phosphate buffered saline (PBS), and treated with a solution of trypsin/ethylenediaminetetraacetic acid (EDTA) in PBS to detach cells from the flasks. After cells were detached, an equal volume of fresh medium was added to inhibit the trypsin activity. The cells were re-suspended in media and cell density was determined with a hemacytometer. Cells were replanted in fresh medium in new tissue culture flasks for continued culture and multi-well tissue culture plate for experiments. In the case of the cell proliferation assays to be described below the suspension was diluted to a concentration of 40,000 cells per milliliter. 100 µl of the cell suspension is added to each well of 96-well plates (except for the first column which were left empty) which were then placed in the incubator. In the case of the wound closure assays to be described below 500 µl of cell suspension was added to each well of 24-well plates which were then placed in the incubator.

Wound Closure Assays

The 24-well plates described previously were incubated until cells were confluent and forming a monolayer as determined by light microscope. The media in the wells of the other plates were decanted and replaced with fresh media containing varying concentrations of DX-52-1 analog. Different plates were designated for different DX-52-1 Analogs and the columns on each plate were each designated for a different concentration (control (0 nM [control], 62.5 nM, 125

nM, 250 nM, 500 nM and 1 μM). The plates were then incubated for an additional 30 minutes. Wounds were scratched into the center of each well using disposable pipette tips. These wounds were photographed using a digital camera mounted on a light microscope. The plates were then incubated over a 48 hour period with the wounds in each well being photographed at 3 hrs, 6 hrs, 9 hrs, 12 hrs, 24 hrs, and 48 hours. The perimeter of each would was measured in each photograph by using ImageJ software. The percent wound closure at each time point was defined as (1 – wound size/initial wound size) * 100%.

Cell proliferation Assays

The 96-well plates described previously were incubated for 24 hours. The cell population of one plate was counted with the Cell Counting Kit-8 (Dojindo), a tetrazolium salt-based assay, with an absorbance plate reader (SpectraMax Plus^{384}, Molecular devices) based on manufacturer's protocol. The media in the wells of the other plates were decanted and replaced with fresh media containing varying concentrations of DX-52-1 analog. Different plates were designated for different DX-52-1 Analogs and the columns on each plate were each designated for a different concentration (control (0 nM [control], 62.5 nM, 125 nM, 250 nM, 500 nM and 1 μM). The plates were further incubated for 48 hours. The cell populations of each plate were quantified with the Cell Counting Kit-8 (Dojindo) as previously described. The effects of the DX-52-1 analogs on cells proliferation were computed by dividing the average cell counts in each column of each plate by the average of cell count of the plate incubated only 24 hours before quantification. The ratios indicated the effect of the various DX-52-1 analogs on cell proliferation over a 48 hour period.

Results and Discussion

In the competition assays of DX-52-1 and its assorted derivatives vs. BDX in regards to Galectin-3, none of the derivatives had band intensities their lanes equivalent to the band intensity present in the control lane (Figure 4-2A). This would indicate that none of the functional group modifications completely prevented competition and therefore all the derivatives were able to bind to Galectin-3. However most of the band intensities of the derivatives were greater than the band intensity in the DX-52-1 lane (Figure 4-2A) which suggests in most of the functional modifications caused reductions in competition as computed by Equation 2 (Figure 4-2B) and most likely binding ability as well. The derivative BO1-95 which involves a substitution of the DX-52-1 hydroxyl group with an acetyl group, exhibited a competition of 47.7% ± 28.6% (p value < 0.05) when compared with DX-52-1. The derivative BO2-7 which involves the placement of a bromine atom on the DX-52-1 phenyl ring in the para position to the methoxy group, showed no statically significant reduction in competition as compared with DX-52-1. The derivative BO1-180 which combines the functional group modifications of BO1-180 and BO2-7, exhibited a competition of 35.7% ± 1.88% (p value < 0.001). In the case of the derivative 1X-108 which involves the removal of the nitrile group from DX-52-1 showed a competition of 3.76% ± 4.08% (p value < 0.001). The derivative 1X-140 which has the DX-52-1 nitrile group substituted with an ethynyl-TMS group showed a competition of 4.50% ± 1.31% (p value < 0.001). The derivative 1X-141 which has the DX-52-1 carboxyl group substituted reduced to a methyl hydroxyl group showed a competition of 3.51% ± 1.10% (p value < 0.001). The results of the BDX competition assay of DX-52-1 and its derivatives shown a similar pattern in the case of Radixin (Figures 4-3A and 4-3B). BO1-180 and BO1-95 respectively show competitions of 25.6 ± 8.16% and 52.5% ± 1.14% (both p values < 0.001) relative to DX-52-1. BO2-7 showed no a relative competition that did not differ from DX-

52-1 in a statistically significant way. The relative competitions of 1X-108, 1X-140, and 1X-141 were respectively 22.2% ± 12.7%, 16.5% ± 6.37%, and 22.8% ± 14.5% (p value < 0.001 in all three cases).

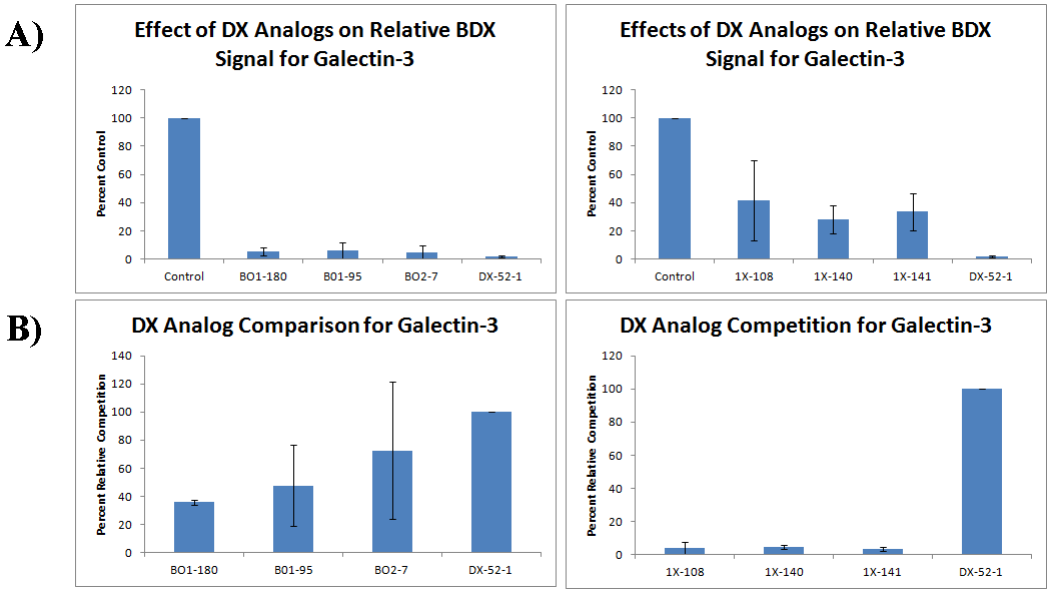


Figure 4-2: BDX Competition Assays of DX-52-1 and its Assorted Derivatives with Regard to Binding Galectin-3. Measurements were made by western blots using antibiotin-HRP to probe for BDX

A) BDX Signal Intensity as a Percentage of the BDX Signal Intensity in the Control Lane Relative Intensity is defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Galectin-3 Control Sample (SD, $n = 3$).

B) Percent Competition Relative to DX-52-1 as Defined by Equation 2 (SD, $n = 3$). All analogs with the exception of BO2-7 showed statistically significant less competition with BDX when compared to DX-52-1 ($p < 0.05$).

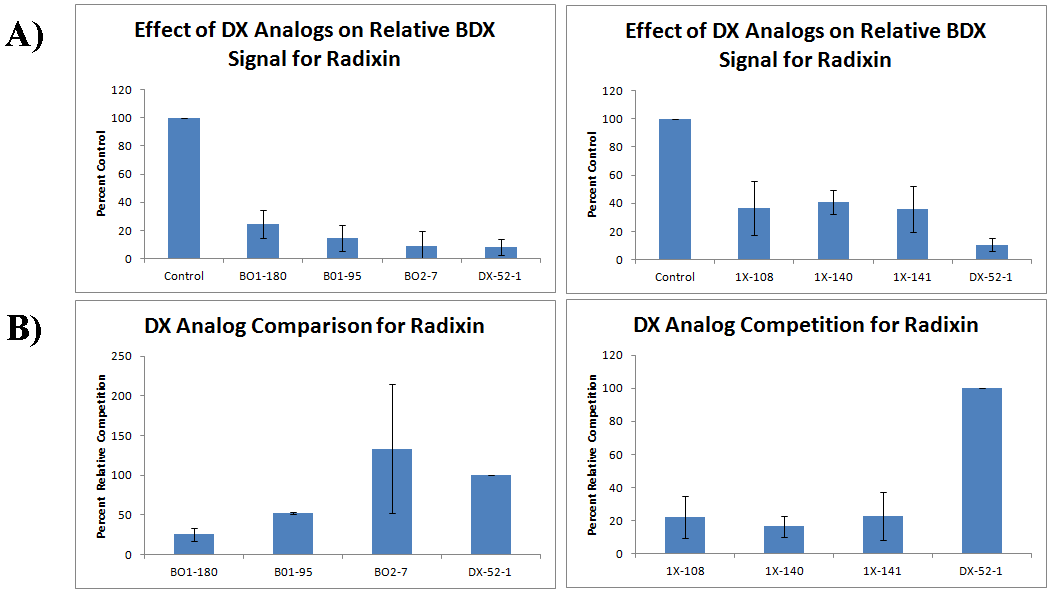


Figure 4-3: BDX Competition Assays of DX-52-1 and its Assorted Derivatives with Regard to Binding Radixin. Measurements were made by western blots using antibiotin-HRP to probe for BDX

A) BDX Signal Intensity as a Percentage of the BDX Signal Intensity in the Control Lane Relative Intensity is defined as the ratio of the directly measured signal intensity of the sample to the directly measured signal intensity of the Radixin Control Sample (SD, n = 3).
B) Percent Competition Relative to DX-52-1 as Defined by Equation 2 (SD, n = 3). All analogs with the exception of BO2-7 showed statistically significant less competition with BDX when compared to DX-52-1 ($p < 0.01$).

These results when considered with the competition assay results for Galectin-3 imply that the hydroxyl, carboxyl, and nitrile functional group of DX-52-1 are important to its ability to binding to both Galectin-3 and Radixin. This might hold true for any other protein that DX-52-1 binds. Furthermore the similarities in the BDX competition assay results suggest the possibility that the binding site in both protein might turn to be similar. If the specific binding of DX-52-1 to Galectin-3 and Radixin is due to hydrogen bonding and/or a salt bridge then that could conceivably explain the important of the hydroxyl and carboxyl group respectively. Kahsai et al had proposed that the mechanism for DX-52-1 bonding is the ejection of the nitrile group resulting in a carbocation that is then attacked by a nucleophilic amino acid side chain on a protein (12). The results for the BDX

competition assays for 1X-108 with Radixin and Galectin-3 mostly support that proposed mechanism. However the mechanism would predict a complete loss of competition with BDX. Perhaps there is a minor secondary mechanism that permits some competition with BDX to be retained. The placement of bromine on the DX-52-1 phenyl ring in the para position to the methoxy group did not seem to reduce the ability of DX-52-1 to compete with BDX in the case of BO2-7. There was no statistically significant difference between the results of BO1-180 and BO1-95 so the bromine had no effect in that case either. It can be concluded that the position on the phenyl group para to the methoxy group is of minimal if any importance to the binding ability of DX-52-1, at least in regard to Galectin-3.

The results of the wound closure assays on MDA-MB-435 cells support the conclusions derived from the BDX competition results all the more strongly. With the sole exception of BO2-7 every single derivative showed no anti-migratory effect on MDA-MB-435 cells at any of the doses tested (Figure 4-4). BO2-7 on the other hand showed a strong anti-migratory effect as a function of dose. In fact the dose response is similar to that of DX-52-1 on MDA-MB-435 cells (Fenteany Group Unpublished Results). The fact that these results point in the same direction as the results from the BDX competition as assay in terms of which functional groups are important, strongly suggest that the anti-migratory effect of DX-52-1 is brought about by its interaction with Galectin-3 and Radixin. Furthermore this convergence of results between wound closure and BDX competition assays support the proposition that Galectin-3 and Radixin play critical roles in cell motility.

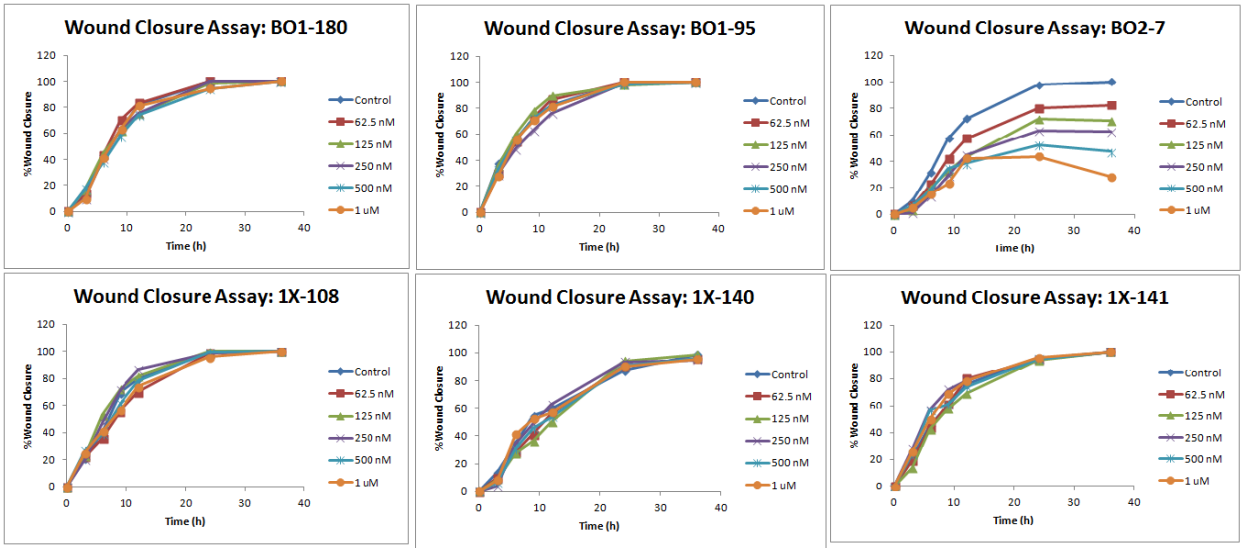


Figure 4-4: Wound Closure Assays of MDA-MB-435 over a 36 period with DX-52-1 Derivatives. Concentrations range from 0 to 1µM. With the notable exception of BO2-7 all analogs show no visible reduction in rate of wound closure. The results for BO2-7 mirrors unpublished Fenteany Group results concerning the effect of DX-52-1 on MDA-MB-435 wound closure.

This converge of results is further joined by the outcome of the cell proliferation assays performed on MDA-MB-435. Only BO2-7 showed a substantial reduction in the proliferation of MDA-MB-435, while the other derivatives did not show a statistically significant difference from their respective controls. (Figure 4-5). It can be surmised from the proliferation assay results that the anti-proliferation properties of DX-52-1 upon MDA-MB-435 can be attributed to the Galectin-3 and Radixin and as such those two proteins are likely to play a critical role in cell proliferation. Kahsai et al had come to similar conclusion when they demonstrated that over-expression of Galectin-3 counteracted the effect of DX-52-1 on cell motility (13).

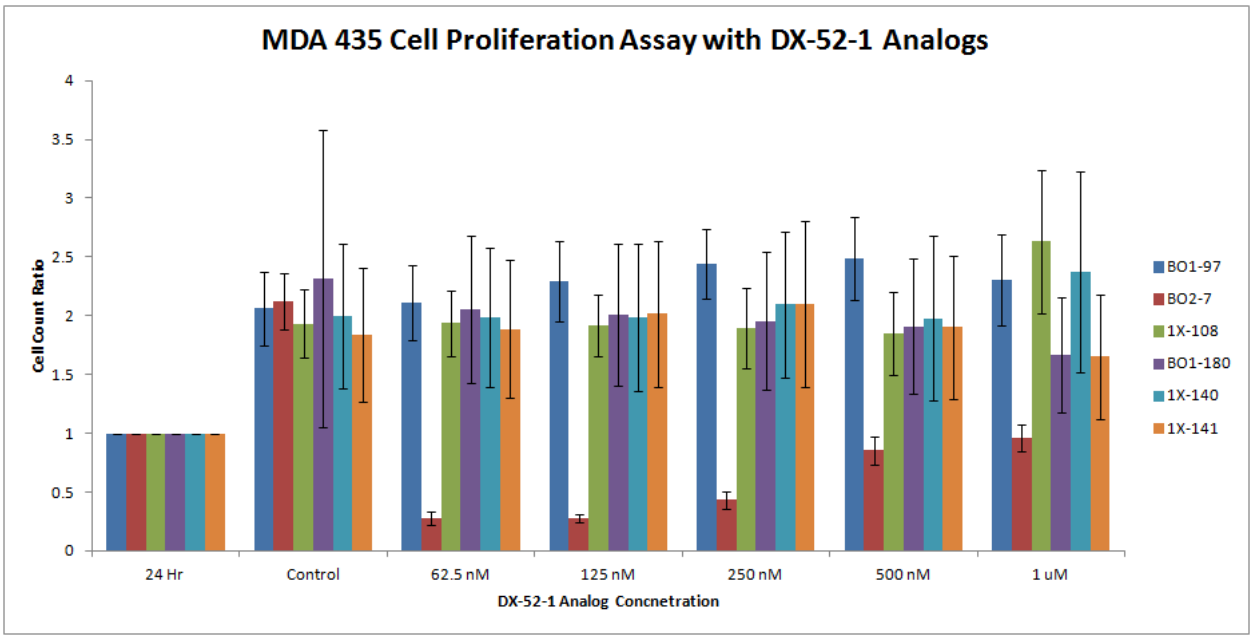


Figure 4-5: Cell Proliferation Assays of MDA-MB-435 over a 36 period with DX-52-1 Derivatives. The concentrations of each derivative range from 0 to 1µM (SD, *n* = 6). Only BO2-7 shows substantial reductions in cell count at any concentration. In fact BO2-7 shows a substantial reduction in cell even at the lowest concentration tested. The results for BO2-7 mirrors unpublished Fenteany Group results concerning the effect of DX-52-1 on MDA-MB-435 cell proliferation.

The successful treatment of cancer depends on the ability to inhibit the proliferation and migration of tumor cells. The SAR results from the BDX competition, wound closure and proliferation assays have shown the DX-52-1 phenyl group could be modified to some extent without seriously impairing the binding properties of DX-52-1 or its anti-migratory and anti-proliferation properties. Even if DX-52-1 itself cannot be used as such treatment it has successfully demonstrated that Galectin-3 and Radixin are critically important in such an enterprise. Furthermore the demonstrated importance of the hydroxyl, carboxyl, and nitrile groups could provide some insight in how to design other possible anti-cancer drugs.

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